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Characterization of mitochondrial respiratory chain defects using DNA microarrays

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CONTENTS

SUMMARY

Respiratory chain disorders classically result from the lack of activity of one or more specific enzymes or defects in the protein transport. These disorders represent a wide group of mainly severe disorders with a broad range of pathological states that vary in age of onset, severity, and phenotypic presentation. Their diagnostic workup has to be complex, and novel genetic techniques and genomic approaches are needed to streamline the process. One of those powerful genomic tools for qualitative and quantitative analysis of nucleic acids is a microarray technology. This technology is widely applied not only in primary research, but also in clinical diagnostic and could be used in various modifications such as expression analysis, detection of polymorphisms, comparative genomic hybridization or mutation detection.

This thesis demonstrates the development and application of microarray technology in different studies, all aimed at characterization and identification of disease causing genes. Namely, presented studies were focused on understanding of mitochondrial inherited disorders with nuclear genetic origin (ATP synthase deficiency), characterization of altered mitochondrial biogenesis in renal carcinoma cells, and on molecular characterization of mucopolysaccharidosis IIIC. All results prove the usefulness of microarray technology in biomedical research and, in particular, in identification of molecular basis of inherited metabolic disorders.

SOUHRN

Poruchy respiračního řetězce jsou zpravidla způsobeny ztrátou aktivity jednoho případně několika specifických enzymů nebo poruchou transportu proteinů. Tyto poruchy představují širokou skupinu onemocnění s rozsáhlým spektrem patologických stavů, které se liší věkem nástupu onemocnění, závažností a klinickým fenotypem. Jejich diagnostika musí být komplexní a vyžaduje využití nových genetických a genomických technik, které tento proces urychlují a zefektivňují. Jedním z těchto genomických nástrojů pro kvalitativní a kvantitativní analýzu nukleových kyselin je technologie DNA čipů. Tato technologie je nyní využívána nejen v základním výzkumu, ale také v klinické diagnostice a to v několika modifikacích, jako je analýza genové exprese, detekce polymorfismů, komparativní genomová hybridizace nebo detekce mutací.

Tato dizertační práce se zabývá vývojem a následnou aplikací čipové technologie ve studiích zaměřených na identifikaci a charakterizaci genů způsobujících závažná onemocnění. Realizované projekty byly zameřeny především na porozumění mitochondriálním dědičným poruchám jaderného původu (poruchy ATP syntázy), na charakterizaci změn v mitochondriální biogenezi v ledvinných nádorových buňkách a na molekulární charakterizaci mukopolysacharidózy IIIC. Všechny výsledky dokumentují významnost čipové technologie v biomedicínském výzkumu a při identifikaci molekulární podstaty dědičných metabolických poruch.

1 INTRODUCTION

A mitochondrion is an organelle under nuclear and mitochondrial genetic control and its maintenance requires a nucleo-mitochondrial cross-talk. Better understanding of mitochondrial communication and regulation could be accomplished by studying of mitochondrial pathologies using various gene expression techniques. This thesis presents the implementation and application of microarray technology for identification of disease causing genes in respiratory chain disorders, especially in patients with ATP synthase deficiency of nuclear origin. The introduction to this thesis provides an overview of mitochondrial structure and biogenesis, respiratory chain complexes and their disorders as well as different types of microarrays. A study of mitochondrial ATP synthase deficiency represents the major experimental part of this thesis and is accordingly introduced in greater detail. This study led to setting up the methods for microarray technology that were used in further studies centered on characterization of inherited metabolic disorders. This is documented in positional cloning of patients with lysosomal storage disorders - mucopolysaccharidosis IIIC.

1.1 Mitochondria

1.1.1 Mitochondria structure and function

Mitochondria are localized in the cytoplasm of nearly all eukaryotic cells. They are small oval organelles of 0.5 – 1 μm in diameter and several μm in length. The number of mitochondria per cell and their shape differ in various cell types and can change. Each mitochondrion is defined by two highly specialized membranes outer and inner. The space between them defines intermembrane space which contains multiple copies of circular mitochondrial DNA (mtDNA). The eukaryotic cell contains in average 10^3 – 10^4 copies of mtDNA with 2 – 10 genomes per organelle (Nelson and Cox, 2005). The main mitochondrial function is to generate most of the cellular energy in the form of ATP (adenosine triphosphate). Mitochondria also play a pivotal role in other processes such as cell differentiation, regulate cellular redox state or cytosolic concentration of Ca^{2+}. Mitochondria are a source of endogenous reactive oxygen species (ROS), and integrate many of the signals for initiating apoptosis. Therefore the mitochondrial function is strictly controlled at the cellular level (Ackerman and Tzagoloff, 2005; Cannino et al., 2007; Garesse and Vallejo, 2001).

1.1.2 Mitochondrial genetics and biogenesis

A mitochondrial genome consists of a double strand covalently closed circular DNA molecule (16 569 base pair in mammals). The two strands are named after their buoyant density heavy (H) and light (L). Many mtDNA molecules are packaged within mitochondria into small clusters called nucleoids (Jacobs et al., 2000) that vary in size and number. Mitochondrial DNA consists only of a small number of genes. Mammals have 22 tRNA, 2 rRNA and 13 genes coding a set of the inner mitochondrial membrane proteins, all of them component of oxidative phosphorylation complex (OXPHOS). Both the mitochondrial replication and transcription take place in the organelle. The mitochondrial genome replicates in a relaxed fashion some mtDNA molecules undergo multiple rounds of replication while others do not replicate. Transcription of mtDNA is polycistronic and completely dependent on nucleus-encoded gene products. Mitochondrial protein coding genes and rRNA genes are interspersed with tRNA genes that are thought to demarcate the cleavage site of RNA processing. Mitochondrial polycistronic transcripts are processed to monocistronic and further translated on mitochondrial ribosomes bound to the matrix side of the inner membrane, and cotranslationally inserted into the proper compartment (Allen et al., 2005; Scarpulla, 2006). All the other mRNAs for mitochondrial proteins are transcribed in the nucleus (Saraste, 1999) and translated by cytoplasmic ribosomes. Proteins are then imported into mitochondria and distributed to appropriate compartments: inner and outer membrane, matrix and intermembrane space (Poyton and Dagsgaard, 2000).

Mitochondrial biogenesis and physiology depend on the coordinate expression of about 1500 different proteins. The mitochondrion contains approximately 80 protein components of the oxidative phosphorylation system, of which 13 are encoded by mtDNA and the rest by nuclear genome (Meisinger et al., 2008; Pagliarini et al., 2008). Biogenesis, maintenance and functional regulation of mitochondria therefore require communication and coordination between these two genomes which is executed at several levels such as protein-protein interactions or protein-nucleic acid interactions.

Transcription regulators acting on both nuclear and mitochondrial respiratory chain genes play an important role in nucleo-mitochondrial cross-talk. A mitochondrial transcription is directed by a small number of nucleus-encoded factors (Tfam, TFB1M, TFB2M, mTERF). The expression of these factors is coordinated through the action of transcriptional activators and coactivators. In particular, environmental signals induce the expression of PGC-1 family coactivators (PGC-1α, PGC-1β and PRC), which in turn target

specific transcription factors (NRF-1, NRF-2 and ERR) in the expression of respiratory genes (Scarpulla, 2002; Scarpulla, 2006; Vercauteren et al., 2006). Transcription regulators interact with mitochondrial proteins mRNAs thereby influencing their metabolism and expression. A tight regulation of the import and final assembly of mitochondrial proteins is essential to endow mitochondria with functional complexes. A mechanism involved in the nucleus-mitochondrion cross-talk plays an essential role in the metabolism of the whole cell (Cannino et al., 2007; Garesse and Vallejo, 2001; Nisoli et al., 2004; Scarpulla, 2002; Spinazzola and Zeviani, 2007).

1.1.3 Oxidative phosphorylation system

The production of energy in the form of ATP is an essential process for all cells. In oxidative phosphorylation, the electrons from oxidized substrates are stored in the form of redox equivalents NADH and $FADH_2$ that are utilized by a series of redox enzyme complexes located within the mitochondrial inner membrane (electron transport chain or respiratory chain). The electrons enter at either complex I (NADH) or II ($FADH_2$) and are transferred through the coenzyme Q to complex III, then to the cytochrome *c*, to complex IV, and finally to oxygen to generate H_2O. The transfer of electrons through the protein complexes to molecular oxygen and the translocation of protons generate a proton gradient across the inner mitochondrial membrane. This gradient is then used by complex V, ATP synthase, to generate ATP and also to support the metabolite and protein transport across the inner membrane. ATP generation also involves other integral membrane proteins, phosphate transporter and ADP/ATP carrier (AAC) that is responsible for supply of ATP to the rest of the cell and for replenishment the mitochondrial matrix with ADP (Klingenberg, 2009; Pebay-Peyroula et al., 2003). The assembly, enzymology and regulation of mitochondrial OXPHOS have been studied for many years, but its physical organization is still contentious.

1.1.4 Complex V - ATP synthase structure and biogenesis

The mitochondrial ATP synthase is a heterooligomeric complex. It is a key enzyme of mitochondrial energy provision, and catalyzes the synthesis of ATP during oxidative phosphorylation. The ATP synthase is a 650 kDa protein complex composed of 16 types of subunits (Fig. 1), which form two main parts: F_1 and F_o. Six subunits form the globular F_1 catalytic part (α, β, γ, δ, ε, and loosely attached inhibitory factor 1 - IF1). F_o functions as a proton channel and consists of additional ten subunits (a, b, c, d, e, f, g, OSCP, A6L, and F_6)

(Collinson et al., 1996). Two mammalian ATP synthase subunits a (*ATP6* gene) and A6L (*ATP8* gene) are encoded by mtDNA; all the others are encoded by nuclear DNA. F_1 is connected to the membrane-embedded F_o portion by two stalks. In bovine heart, the central stalk is composed of subunits δ, γ, ε and the peripheral stalk consists of subunits OSCP, d, F_6, and the soluble part of subunit b (Abrahams et al., 1994; Collinson et al., 1994). Oligomer of subunits c represents a rotor of the enzyme. Electron crystallography and mass spectrometry have shown that the c-ring of F_1F_o ATP synthase can contain different numbers of subunit c: c_{10} in yeast and mammals, c_{11} and $c_{13\text{-}15}$ in bacteria and c_{14} in chloroplasts. A flow of protons, down the gradient, through the F_o motor drives a rotation of the c-ring rotor connected to subunit γ. The rotation of subunit γ causes conformational changes in the catalytic nucleotide-binding sites on the three β subunits of the F_1 motor, resulting in synthesis and release of ATP.

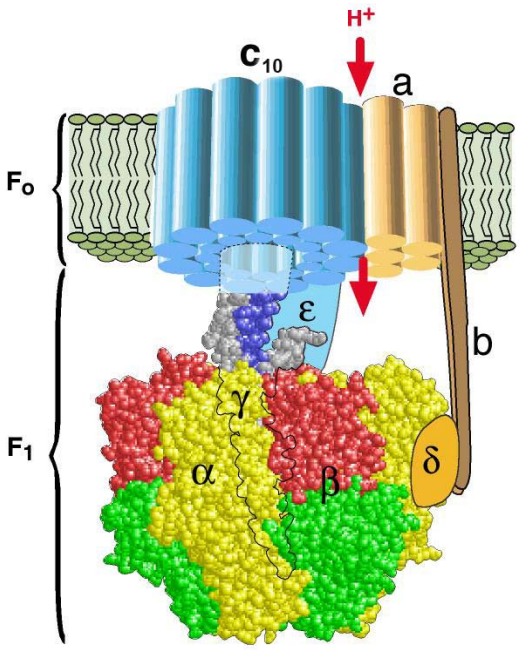

Figure 1: ATP synthase structure and function. The mitochondrial ATP synthase (complex V) consists of two multisubunit components, F_o and F_1, which are linked by two stalks. F_o spans the lipid bilayer, forming a channel through which protons can cross the membrane. Proton movements through F_o down the electrochemical gradient drive conformation changes in F_1 via rotation of inner stalk subunit c-γ-δ-e resulting in phosphorylation of ADP. Adapted from (Wang and Oster, 1998).

ATP synthase biogenesis

The ATP synthase complex is formed stepwise with the assistance of several assembly factors, but the mechanism of the mammalian ATP synthase assembly from individual subunits is still not well understood. In the case of F_1, there is a close similarity of its structure in all types of organisms (mammalian cells, lower eukaryotes and prokaryotes). Concerning

the F_o, the situation is complicated by increasing evolutionary complexity of the F_o structure, which gained 7 new subunits from bacteria to man. Most of the present knowledge of mitochondrial ATP synthase biogenesis originates from studies in yeast, but the assembly process in the mammalian cell might be modified as there are substantial differences between higher and lower eukaryotes such as the number and location of F_o subunit c genes, ATP synthase-specific assembly factors, or factors regulating transcription of mtDNA-encoded ATP synthase genes (Houstek et al., 2006). The eukaryotic ATP synthase is formed stepwise and the enzyme assembly begins with formation of the F_1 catalytic part which then associates with the central stalk and subunits c, which oligomerize to form a 10 subunits ring. The c-F_1 pre-complex is thought to be further completed by addition of peripheral stalk, subunit A6L, and other component of F_o part, and finally the subunit a. The two mtDNA encoded subunits a and A6L are added at the last step of the enzyme assembly. These subunits prevent the formation of partially assembled enzymes (Fig. 2) (Houstek et al., 2006; Nijtmans et al., 1995).

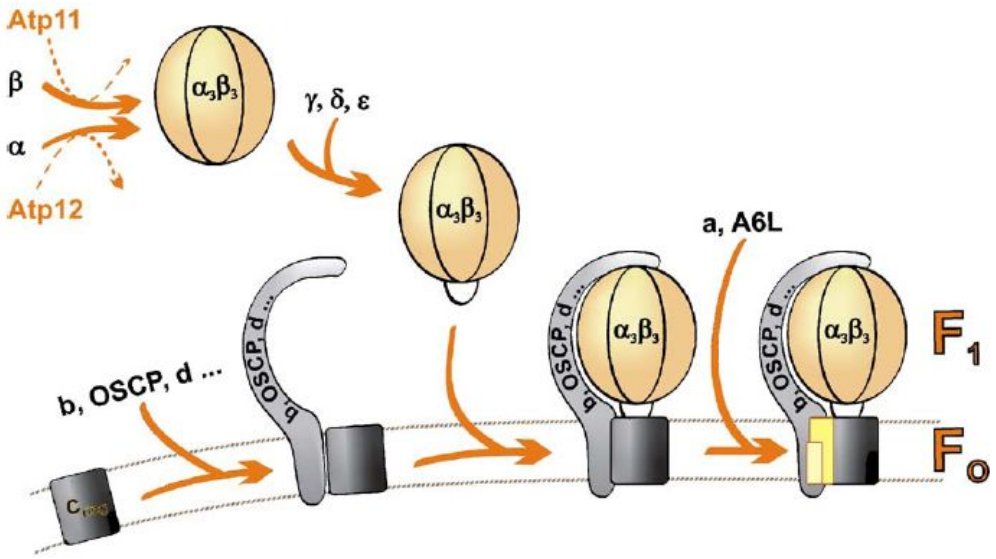

Figure 2: Assembly of the mammalian ATP synthase. ATP synthase is formed stepwise. Assembly of the F_1-part is dependent upon the assistance of specific ancillary factors, ATP11 and ATP12. In the yeast enzyme, additional factors have been described to be involved in the formation of F_1 (ATP11, ATP12, FMC1) and F_o (ATP10, ATP23). Other yeast factors are involved in processing of mtDNA encoded subunits (NCA1-3, NAM1, AEP1-3, ATP22, ATP25) (Houstek et al., 2009; Houstek et al., 2006).

The biogenesis of eukaryotic ATP synthase requires a concerted action of several helper proteins with chaperon-type functions, specific to the assembly of ATP synthase. There are also other important proteins that govern the expression of ATP synthase genes at

different levels. This process is well characterized in yeast where several of these factors involved in F_1 and F_o assembly were described, but only a few have been found in mammals.

Mitochondrial F_1-ATPase contains a hexamer of alternating α and β subunits. The assembly of this structure requires two specialized chaperones, ATP11 and ATP12 that bind transiently to β and α, respectively. In the absence of ATP11 and ATP12, the hexamer is not formed, and β and α precipitate as large insoluble aggregates. Absence of another protein FMC1 also results in the aggregation of the α-F_1 and β-F_1 proteins. Therefore it was proposed that FMC1 assists the folding/stability or functioning of ATP12 (Lefebvre-Legendre et al., 2001). Importantly, as long as ATP11 is bound to β and ATP12 is bound to α, the two F_1 subunits cannot interact at either the catalytic site or the noncatalytic site interface. Only for ATP11 and ATP12 assembly factors, functional homologues in mammals were found (Ackerman and Tzagoloff, 2005; Ludlam et al., 2009; Pickova et al., 2005; Wang et al., 2001). F_o part assembly and formation is assisted by ATP10 and ATP23 where ATP23 is a 32 kDa metallopeptidase of the inner mitochondrial membrane. ATP23 has a dual activity in yeast: maturation of the mitochondrial-encoded subunit 6 after its insertion into the inner membrane, and promotion of the association of mature subunit 6 with subunit 9 oligomers. This assembly step is thus under the control of two substrate-specific chaperones, ATP10 and ATP23, which act on opposite sides of the inner membrane (Osman et al., 2007). A mammalian ortholog of ATP23 contains a HEXXH motif of the protease active site, but its function is unknown.

There are other proteins that govern the expression and translation of ATP synthase genes and subunits, respectively. Processing and stability of subunits 6 and 8 are controlled by NAM1, AEP3, NCA2, NCA3 (Camougrand et al., 1995; Pelissier et al., 1995; Wallis et al., 1994) and translation of subunit 6 (*ATP6*) mRNA is regulated by ATP10, ATP23, and ATP22 (Zeng et al., 2007). ATP25 is a novel RNA stabilization factor specific for the 0.95 kb *ATP9* (subunit 9) mRNA (Zeng et al., 2008). A similar function was described in NCA1, AEP1 and AEP2/ATP13 proteins (Barbash, 2008), which are still known only in yeast.

In 2004 De Meirleir et al. identified a homozygous missense mutation in the third exon of the *ATP12* gene in a patient with severe encephalopathy. It was confirmed that the primary defect was in the assembly of the F_1 catalytic part of ATP synthase, due to dysfunction of ATP12 assembly factor (De Meirleir et al., 2004). Within the last several years, increasing numbers of patients with nuclear genetic ATP synthase defects have been found. Most of them present with early onset lactic acidosis, cardiomyopathy, variable CNS involvement and 3-methylglutaconic aciduria (Houstek et al., 2006; Sperl et al., 2006).

Therefore many attempts have been made to uncover the pathogenic mechanism of ATP synthase deficiency. Recently, the group of 13 patients with ATP synthase deficiency has been analyzed by microarray transcription profiling (Cizkova et al., 2008a). Consequently homozygosity profiles were intersected with whole genome expression profiles determined in patient fibroblast. As a result, gene *TMEM70* on chromosome 8 was identified as a highly probable candidate, and subsequently shown to be mutated (Cizkova et al., 2008b). Pathogenicity of this mutation was confirmed in complementation experiments. The exact function of TMEM70 is still unknown, but it is clear that TMEM70 represents a novel type of eukaryotic ancillary factor involved in the biogenesis of ATP synthase. TMEM70 appears to be responsible for a vast majority of cases of ATP synthase deficiency of nuclear genetic origin (Cizkova et al., 2008b; Houstek et al., 2009).

Supramolecular organization of ATP synthase

ATP synthase biogenesis includes the generation of dimers with the aid of subunits e and g, formation of higher oligomers (Wittig and Schagger, 2009) and possibly also supercomplexes with other inner mitochondrial membrane proteins, e. g. with phosphate and adenine nucleotide carriers in the "phosphorylating assembly" - so called ATP synthasome (Chen et al., 2004; Ko et al., 2003; Pedersen, 2007).

The dimeric assembly state of the F_1F_o-ATP synthase has been demonstrated in mitochondria isolated from diverse organisms such as yeast, algae, plants and mammals. The current evidence indicates that the ATP synthase dimers can further organize themselves into tetramers and larger oligomeric complexes, which then can form a linear and regular array of oligomers within the mitochondrial inner membrane (Stuart, 2008; Thomas et al., 2008). Double rows of F_1 form a helical array around the cristae and could be therefore responsible for the cristae formation (Vonck and Schafer, 2009). ATP synthase dimers isolation has shown the presence of two dimer-specific subunits of yeast ATP synthase, subunit e (Su e; Atp21) and subunit g (Su g; Atp20), which were not essential for catalysis (Arnold et al., 1998; Wagner et al., 2009). Both the e and the g subunit are F_o components with a single transmembrane span (Stuart, 2008). Homodimers of e are associated with the formation of higher oligomers (Arselin et al., 2003) whereas e - g heterodimers are responsible for the formation of ATP synthase dimers (Arselin et al., 2003). In all cases the dimers were found to be associated through their F_o domains, which are at angles of 35 - 90° to each other, thus bending the membrane. The angled arrangement of the F_o parts strongly supports the notion that this association between the ATP synthases is responsible for inner membrane

morphology and cristae formation, but molecular details of the dimer interactions are still unclear (Vonck and Schafer, 2009). Presence of subunits e and g is important for optimal COX activity, and the correct organization of the cytochrome bc_1-COX supercomplex and its association with the TIM23 machinery (Saddar et al., 2008).

Another factor involved in regulation of ATP synthase dimerization is IF_1 (inhibitor factor 1), a 10 kDa basic protein (Campanella et al., 2009). When the transmembrane proton gradient is not generated by the respiratory chain, F_1F_o hydrolyses ATP and pumps protons out of the matrix. To prevent ATP hydrolysis, IF_1 binds at the α/β catalytic sites of ATPase. IF_1 dimer binds simultaneously two F_1. Under conditions of normal generation of the respiratory electrochemical proton gradient, resulting in pH increase in the matrix space, IF_1 dissociates from the catalytic sites and ATP synthesis takes place normally. Dimerization of ATP synthase was shown to be independent on the inhibitor protein, both in yeast (Dienhart et al., 2002) and bovine heart mitochondria (Tomasetig et al., 2002). Therefore it appears that function of IF_1 *in vivo* is binding to preexisting ATP synthase dimers (Zanotti et al., 2009).

1.2 Mitochondrial diseases

Mitochondrial myopathies (also known as "mitochondrial encephalomyopathies") are a broad group of disorders first described in the early 1960s. An estimated birth incidence for mitochondrial diseases is about 1:5000 (Thorburn, 2004). Up to now in humans, several hundreds of diseases resulting from various defects of mitochondria biogenesis, defects of respiratory chain complexes or defects of individual mitochondrial proteins have been reported. Defects are usually associated with a vast array of multisystemic disorders. These disorders are especially interesting from the genetic point of view because the respiratory chain is the only metabolic pathway in the cell that is under the dual control of the mitochondrial genome and the nuclear genome. According to recent data obtained from integrative analysis, the mitochondrial proteome is nowadays estimated to consist of approximately 1500 proteins (Meisinger et al., 2008; Pagliarini et al., 2008). These proteins play an essential role in the assembly or maintenance of individual OXPHOS complexes, in mtDNA integrity and in mitochondrial biogenesis. The majority of the nuclear origin genetics diseases are inherited as autosomal recessive trait and produce severe and usually fatal phenotype in infants (Shoubridge, 2001a).

An insufficient or altered function of mitochondrial oxidative phosphorylation system represents the most frequent group of human mitochondrial diseases, a broad range of

pathological states that vary in age of onset, severity and phenotypic presentation (DiMauro, 2004; Muravchick, 2008). Underlying genetic defects include mutations both in mitochondrial and nuclear DNA. While the mtDNA mutations frequently affect adult populations, nuclear genetic defects are usually associated with early onset (Shoubridge, 2001b). Mitochondrial dysfunction has also been shown to play a role in the pathogenesis of late-onset neurodegenerative disorders, such as Parkinson, Alzheimer or Huntington diseases and especially in the most common human disease - process of aging (Wallace, 2005).

1.2.1 Mitochondrial diseases caused by mtDNA mutations

Physical characteristics and mode of inheritance of mtDNA define some rules of "mitochondrial genetics" (DiMauro and Schon, 2003). Six aspects of their behavior are critical for understanding the etiology and pathogenesis of mitochondrial disorders: (1) they are maternally inherited; (2) cells typically contain hundreds of organelles and thousands of mitochondrial genomes; (3) mutations can arise in a mtDNA population, resulting in the coexistence of two or more mtDNA genotypes within a single cell, organ or individual (heteroplasmy); (4) if the mutation is pathogenic, the proportion of mutated molecules in the heteroplasmic population (mutational load) affects the severity of the biochemical defect, but not necessarily in a linear fashion; (5) mtDNA replication and inheritance in lineages of somatic cells is stochastic, often resulting in changes of the mutational loads during the patient life, and in different mutational loads in different cells and tissues (mitotic segregation); (6) because different cell types have different minimal oxidative energy requirements, the level of heteroplasmy and the dynamics of the mitotic segregation play a critical role in determination of the clinical presentation and outcome. A mutation rate of mtDNA is 10 – 20 times higher than of nuclear DNA (nDNA). The reasons for this are: include relatively high levels of ROS in mitochondria, the lack of introns, the lack of protective histones of mtDNA and the absence of proof-reading by mtDNA polymerase.

Mitochondrial DNA defects can be classified into three groups - point mutations, rearrangements of mtDNA and mtDNA depletions. These disorders represent a wide range of clinical syndromes (phenotypes), which are often similar for number of different genetic defects. On the other hand, the same mutation could be manifested differently. This overlay of phenotypical symptoms and different manifestation of the same syndrome make this field of diagnosis difficult.

1.2.2 Mitochondrial diseases caused by nDNA mutations

Disorders due to mutations in nuclear DNA are more abundant, not only because most respiratory chain subunits are nucleus-encoded, but also because a correct assembly and functioning of the respiratory chain require numerous steps that are under the control of nDNA. These steps (and related diseases) include: synthesis of assembly proteins; intergenomic signaling; mitochondrial import of nDNA-encoded proteins; synthesis of inner mitochondrial membrane phospholipids; mitochondrial motility, fusion and fission (DiMauro, 2004). Nuclear genes responsible for disturbance of OXPHOS system can be categorized on a structural and functional level: (1) genes encoding structural components of the OXPHOS system, (2) genes encoding assembly factors, (3) genes involved in the maintenance of mtDNA, and (4) genes involved in mitochondrial system biogenesis.

1.2.3 Isolated deficiency of ATP synthase

Isolated defects of ATP synthase of either nuclear or mitochondrial genetic origin belong to the most severe metabolic disorders of the neuromuscular system and typically manifest in children, very often shortly after birth (Houstek et al., 2006). Mitochondrial mutations have been found in two mitochondrially-encoded subunits of the enzyme (a and A6L) causing defects in H^+ channel function; and in two nuclear genes encoding assembly factors of the enzyme (*ATP12*, *TMEM70*), which affect enzyme biogenesis.

Several point mutations in the *ATP6* gene have been associated with a group of maternally inherited, early onset neurodegenerative syndromes (Schon et al., 2001; Wong, 2007): NARP, MILS and FBSN (familial bilateral striatal necrosis), related disorder to NARP. The most common and the best studied of the pathogenic *ATP6* mutations is T8993G. Patients with this mutation load within the range of 70 - 90 % mainly develop the NARP syndrome, while a level of mutated mtDNA higher than 90 % usually results in the MILS. **NARP** (Neurogenic muscle weakness, Ataxia, Retinitis Pigmentosa) causes retinitis pigmentosa, dementia, seizures, ataxia, proximal weakness, and sensory neuropathy. **MILS** (Maternally Inherited Leigh Syndrome) is a more severe infantile encephalopathy with characteristic symmetrical lesions in the basal ganglia and the brainstem (DiMauro and Schon, 2003; Janssen et al., 2004; Shoffner, 1996). A different kind of disorder is the two-nucleotide micro-deletion (ΔTA9205) affecting the stop codon of the *ATP6* gene and the processing site within the tri-cistronic *ATP8-ATP6-COX3* transcript (Anderson et al., 1981). It is a very rare mutation reported in only two cases (Jesina et al., 2004; Seneca et al., 1996).

Recently, a homoplasmic nonsense mutation in the *ATP8* gene has been detected in a 16-year-old patient with apical hypertrophic cardiomyopathy and neuropathy (Jonckheere et al., 2008).

In 2004, the first mutation in assembly factor of complex V was identified. The homozygous missense mutation in the third exon of *ATP12* gene was found in a single patient with severe encephalopathy (De Meirleir et al., 2004). More than dozen patients with ATP synthase deficiency of nuclear origin have been diagnosed (Sperl et al., 2006) and in most of them mutations in *ATP11* and *ATP12* were excluded and no mutations in structural genes were found. Microarray transcription profiling has been performed in 13 cases with ATP synthase deficiency using both tailored and commercial oligonucleotide DNA microarrays. However, the results did not show any pronounced down-regulation of ATP synthase subunits or other known ATP synthase-related genes (Cizkova et al., 2008a). A further study using integrative genomics resulted in finding probable candidate gene *TMEM70* the sequence analysis of that gene revealed a splice mutation. A substitution c.317–2A>G is located in the splicing site of intron 2, which leads to aberrant splicing and loss of the *TMEM70* transcript. Pathogenicity of TMEM70 mutation was confirmed in the complementation study, where fibroblasts from ATP synthase-deficient patients were transfected with wild type *TMEM70* cDNA (Cizkova et al., 2008b). The *TMEM70* gene consists of three exons and encodes a 260 amino acid protein which contains a conserved DUF1301 domain and two putative transmembrane regions indicating that TMEM70 can be a membrane-associated protein. Using phylogenetic analysis, *TMEM70* homologues were found in genomes of multicellular eukaryotes and plants, but surprisingly not in yeast or fungi. Clinical manifestation in affected individuals is neonatal lactic acidosis, hypertrophic cardiomyopathy and/or variable central nervous system involvement and 3-methylglutaconic aciduria. The disease outcome is severe, and half of affected individuals die in early childhood (Sperl et al., 2006). Mutation in *TMEM70* appears to be responsible for a vast majority of cases of ATP synthase deficiency of nuclear genetic origin, primarily in the Roma population (Houstek et al., 2009). So far, no mutation has been reported in the structural nuclear component of complex V.

1.2.4 Pathogenic effect of cellular hypoxia, free oxygen radicals

Mitochondria are an important source of ROS (reactive oxygen species) within most mammalian cells (Murphy, 2009). This is a consequence of the action of oxidative phosphorylation in the generation of unpaired electrons that interact with O_2 and thus generate superoxide anion radical ($O_2^{\bullet -}$). ROS are generally considered as toxic agents contributing to

mitochondrial damage in a range of pathologies such as lipid peroxidation, cell membrane damage and mtDNA damage and also to aging and cancer (Hervouet et al., 2007). Recent data strongly suggest that ROS are involved in physiological signaling cascades regulating various cellular and organ functions (Afanas'ev, 2007; Droge, 2002; Valko et al., 2007) and also redox signaling from the organelle to the rest of the cell (Balaban et al., 2005; Droge, 2002) with H_2O_2 as a main messenger molecule. The main sites of mitochondrial ROS production have been localized at the level of OXPHOS complex I and complex III (Boveris et al., 1976; Fleury et al., 2002). Therefore mitochondria have vigorous antioxidant defences which include antioxidant enzymes (manganese superoxide dismutase, glutathione peroxidase) and free radical scavengers such as thioredoxin, cytochrome *c* (Butler et al., 1975), coenzyme Q (Beyer, 1992) or vitamin E (Ham and Liebler, 1995) in their reduced forms (Hervouet et al., 2007).

ROS production increases when the electron transport is reduced, which occurs at low respiratory rates (Korshunov et al., 1997), at hypoxia or in pathological situations (Wallace, 2005). The discovery of increased mitochondrial ROS production during hypoxia arose from investigations of HIF-1 (hypoxia-inducible factor 1), which plays a central role in the response of cells to hypoxia (Semenza, 2004; Schofield and Ratcliffe, 2004). HIF-1 is a heterodimer comprising HIF-1α and HIF-1β that translocates to the nucleus and there, in association with other proteins, initiates transcription of a number of genes in response to hypoxia (Semenza, 2009; Schofield and Ratcliffe, 2004). HIF-1α is constitutively expressed, but under normoxia, it is rapidly hydroxylated, marking HIF-1α for rapid degradation by the ubiquitin-proteasome system (Semenza, 2004; Schofield and Ratcliffe, 2004). When the O_2 concentration falls, HIF-1α is no longer degraded, allowing the HIF-1 heterodimer to form and induce the transcription of a series of hypoxia-sensitive genes (Murphy, 2009). These genes are included in several processes such as erythropoiesis, angiogenesis, glycolysis, glucose transport or cell cycle, as well as cytochrome *c* oxidase biogenesis.

1.3 Laboratory characterization of OXPHOS defects

1.3.1 Clinical, biochemical and molecular diagnostic of OXPHOS defects

Mitochondrial diseases represent a good example of coordinated multi-step process leading to diagnosis. These modern diagnostic processes consist of various biochemical, enzymatic, immunohistochemical, and molecular biology methods. The initial laboratory tests are performed on body fluids and isolated blood cells. Based on the results, patients with

strong suspicion of OXPHOS defect undergo skin and muscle biopsy and those tissues are then thoroughly investigated. To distinguish between isolated and combined OXPHOS deficiencies, the diagnostic process starts with biochemical measurements (metabolite analysis) and continues with measurements of selected mitochondrial enzyme activities and activities of individual OXPHOS complexes. The diagnostic procedure continues with the analysis of OXPHOS complex protein composition and determination of the molecular defect origin (mtDNA vs nDNA) using cybrid cell analysis. In the case of mtDNA defect analysis of panel of prevalent mtDNA mutations, analysis of mtDNA deletion and depletion follow. Final steps in the diagnostics represent sequencing of mtDNA and of candidate nuclear genes.

DNA diagnosis is reached mostly in patients having mutation in mtDNA. In patients with nuclear defect the molecular basis of the disease remains often unknown.

1.3.2 Strategies in identification of unknown disease causing genes

A diagnostic process is experimentally demanding and time consuming and in majority of cases leads only to biochemical diagnosis. The molecular basis of the disease, especially in nuclear encoded defects, usually remains unknown. Identification of nuclear gene defects in OXPHOS deficiencies requires a combination of positional cloning, functional complementation and candidate gene analysis. The application of these „standard“ procedures is, however, greatly hampered by limited size of affected families, complexity and overlap of observed diseases phenotypes, difficulties in measurement of biochemical phenotypes *in vitro* and by the existence of number of candidate nuclear genes.

A new method which has a potential to contribute significantly to differential diagnosis and research of nuclear OXPHOS deficiencies is microarray technology, e. g. genotyping, homozygosity mapping, copy number variation and gene expression profiling. The gene expression analysis provides an opportunity to compare normal gene expression profiles with profiles of appropriate pathological stages. It enables us to survey the genome for transcripts whose level is altered in affected tissues. This type of analysis has a potential to provide information on disease specific gene expression profiles (Mootha et al., 2003b; Slonim, 2002), information on potential candidate genes (Mootha et al., 2003a) and information on pathogenic mechanism of the disease (Van Der Westhuizen et al., 2003).

Microarray technology provides a new quality in differential diagnostic processes and is valuable in effective search for candidate genes in so far molecularly uncharacterized not only nuclear OXPHOS defects, and also in studying and understanding of molecular models of OXPHOS biogenesis and metabolism.

1.4 Microarrays

The microarray technology is one of modern and powerful genomics tools which have undergone a rapid evolution. With a widespread interest in large-scale genomic research, an abundance of equipment and reagents is now available to a wide scientific community. It is also documented by a number of publications dealing with microarrays. In 1996 there were only 5 publications in PubMed database; the search for the keyword "microarray" will now return more than 32 000 hits (September, 09).

The microarray technology evolved from Southern blotting. The use of a collection of distinct DNAs in arrays for expression profiling was first described in 1987 (Kulesh et al., 1987). These early gene arrays were made by spotting cDNAs onto a filter paper with a pin-spotting device. The use of miniaturized microarrays for gene expression profiling was first reported in 1995 (Schena et al., 1995). A DNA microarray is now a multiplex technology used in molecular biology, genetics, pharmacology, and especially in medicine.

The microarray technology is based on mutual hybridization between two systems. One system is stationary, fixed on a solid surface (probes), while the second, movable, represents a sample of our interest. In standard microarrays, the probes are attached to a solid surface by a covalent bond to a chemical matrix (epoxy-silane, amino-silane, lysine, polyacrylamide or others). The solid surface can be membrane, plastics, gel or microscopic beads, but the most frequently used is glass. A general description of a common type of DNA microarray experiment can be divided into the following phases: array printing, experimental design, sample preparation, sample labeling, and hybridization. Hybridization is followed by scanning that produces the results as image (16bit TIFF image file), which is converted to probe intensities (Fig. 3).

An indispensable component of all microarrays experiment is bioinformatics which includes: experimental setup, feature extraction and data normalization, standardization, quality control, statistical analysis, functional annotation and data accession. This statistical part represents nearly 80 % of whole microarray experiment.

Microarray studies produce massive quantities of functional genomics data both in input (probes characteristics, chip preparation, sample information, labeling, hybridization, scanning conditions) and in output. Therefore standards for recording and reporting microarray-based genomics data are needed. The Minimum Information About a Microarray Experiment (MIAME), developed by Microarray Gene Expression database group (MGED), describes the minimum information required to ensure that microarray data can be easily

interpreted and that results derived from its analysis can be independently verified (Brazma et al., 2001). At present, there are numbers of public repositories for microarray data, where MIAME compliant data can be stored as a prerequisite for their publication (e. g. Gene Expression Omnibus Database at NCBI).

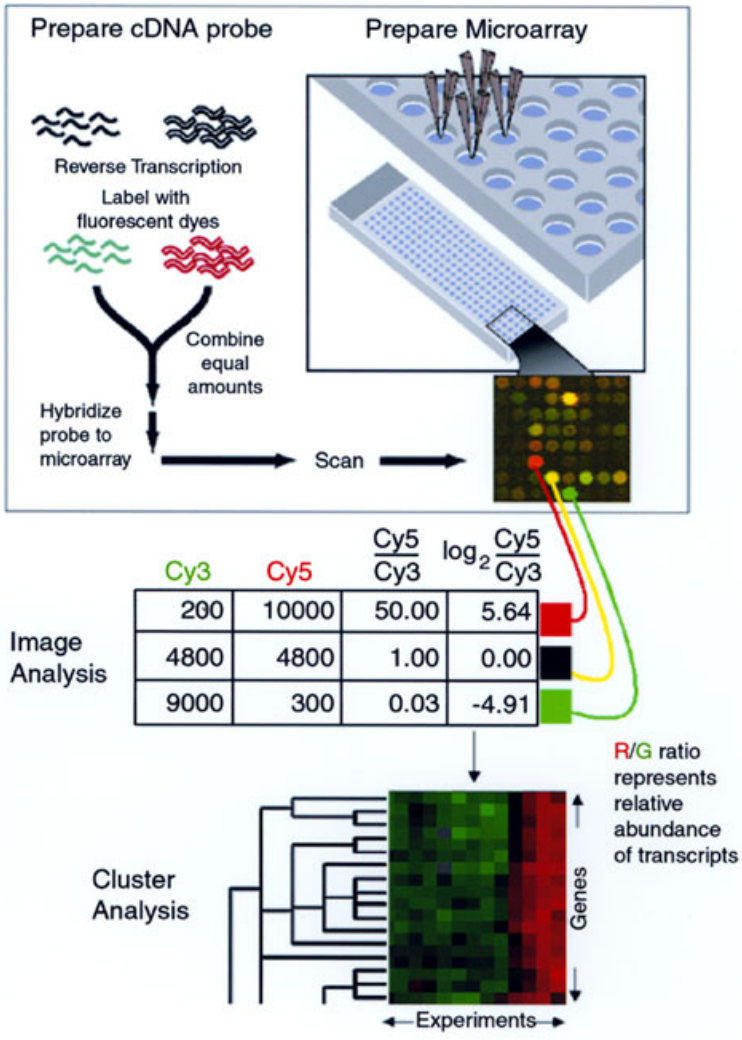

Figure 3: Measuring relative gene expression by using DNA microarrays. Gene expression profiling experiments commonly involve the conversion of RNA or mRNA to cDNA, and labeling of the cDNA with a fluorescent dye for two samples. These are cohybridized to the probes on the array, which is then washed and scanned to detect both fluorophores. In the resulting pseudocolor image, the green Cy3 and red Cy5 signals are overlaid; yellow spots indicate equal intensity for the dyes. After that several steps of cluster analysis is performed. Hierarchical clustering of genes identifies a group of coregulated genes. Adapted from (Cummings and Relman, 2000).

1.4.1 Microarray platforms

All microarrays share a common format based on the analysis of data derived from the affinity capture of biological molecules by ligands. Microarrays are evolving to help expand the understanding of transcriptome complexity: single nucleotide polymorphisms (SNPs),

copy number variation, CpG methylations, microRNAs, or in genomes resequencing. Microarrays also differ in fabrication, workings, accuracy, efficiency, and cost. Additional factors for microarray experiments are the experimental design and the methods of data analyzing. Commercial companies provide custom made arrays for the human genome and a whole host of model organisms. Custom arrays offer an expanded selection of custom solutions, including whole-transcript expression, exon analysis, ChIP-chip, transcript mapping, SNP discovery, 3' expression, resequencing, and whole-genome genotyping. These arrays are customized for the needs of each study and could reduce a vast amount of redundant data.

2 AIMS OF THE THESIS

Respiratory chain disorders represent a wide group of often rare but severe diseases. For effective treatment it is necessary to understand their molecular basis, which demands many modern laboratory techniques. Clearly, new approaches in molecular genetics and genomics need to be applied. One of such methods, which has a potential to contribute significantly to differential diagnostics and research, is gene expression profiling. Therefore the main goals of my work were to develop and optimize conditions for application of microarray technology, which assists to describe ATP synthase deficiency, characterize mechanism of down regulation of mitochondrial biogenesis in renal carcinoma cells, and find the disease causing gene in mucopolysaccharidosis type IIIC.

A) Gene expression profiling using different platforms (h-MitoArray, Agilent)

- Construction, validation and testing of oligonucleotide microarray (h-MitoArray) for gene expression profiling of metabolic disorders, especially respiratory chain defects
- Correlation of h-MitoArray with commercial platform Agilent

B) Characterization of a unique group of patients with ATP synthase deficiency

- Expression profiling of controls/patients with mitochondrial F_1F_o ATP synthase deficiency
- Characterization of pathological mechanisms using pathway analysis
- Positional cloning and identification of disease causing gene

C) Gene expression analysis in cancer cells with down regulation of OXPHOS

- Gene expression study of cell lines deficient in von Hippel-Lindau gene and cell lines complemented by *VHL* gene using custom microarrays

D) Gene expression analysis in mucopolysaccharidosis type IIIC

- Development of custom array for identification molecular basis and disease causing gene of mucopolysaccharidosis type IIIC

3 SUMMARY OF THE RESULTS

The present thesis consists of four publications. The first two publications are concerned with ATP synthase deficiency in mitochondrial diseases, the third publication deals with downregulation of mitochondrial biogenesis in tumorogenesis of renal carcinoma. The fourth publication presents a preparation and application of custom expression array in characterization of lysosomal storage disorder Mucopolysaccharidosis type IIIC.

1. Development of a human mitochondrial oligonucleotide microarray (h-MitoArray) and gene expression analysis of fibroblast cell lines from 13 patients with isolated F_1Fo ATP synthase deficiency,
Čížková, A., Stránecký V., Ivánek, R., Hartmannová, H., Nosková, L., Piherová, L., Tesařová, M., Hansíková, H., Honzík, T., Zeman, J., Divina, P., Potocká, A., Paul, J., Sperl, W., Mayr, J., Seneca, S., Houštěk, J. and Kmoch, S., *BMC Genomics. 2008 Jan 25;9:38.*

This publication represents our first encounter with gene expression analysis of patient fibroblasts with mitochondrial ATP synthase deficiency. Eleven patients were with ATP synthase deficiency of nuclear origin with unknown molecular basis and two patients with mutation in ATP synthase subunit a (*ATP6*). We designed, produced, and validated an oligonucleotide microarray (h-MitoArray) allowing the expression analysis of 1632 human genes (see the list at http://www.biomedcentral.com/1471-2164/9/38/additional/), focused on expression profiling of human mitochondria related genes (mitochondrial biology, cell cycle regulation, signal transduction and apoptosis). The h-MitoArray contained a set of 1632 genes, of which 992 were mitochondrial genes, 42 lysosomal genes, 277 genes associated with apoptosis, and 321 genes known to be involved in carcinogenesis. A set of genes also contained 146 human "housekeeping" genes and 10 *Arabidopsis* genes for normalization and background correction. Using h-MitoArray we searched for gene expression changes in genetically heterogeneous group of 13 patients with F_1F_o ATP synthase deficiency.

A comparison of expression profiles and functional annotation, gene enrichment and pathway analyses of differentially expressed genes defined three subgroups of patient cell lines – M group with mtDNA mutation and N1 and N2 groups with nuclear defect, see Fig 4. It suggested that the underlying biochemical defect has diverse effects on cell gene expression phenotype. In the M group synchronized suppression of mitochondrial biogenesis was found. It could be interpreted as an ATP depletion mediated G1/S arrest (Gemin et al., 2005) associated with synchronized replication arrest of mitochondrial genome (Martinez-Diez et al., 2006). The N1 group showed the elevated expression of some complex I genes and reduced expression among the genes for subunits of complexes III, V, and reduced expression

of genes involved in phosphorylation dependent signalling along MAPK, Jak-STAT, JNK, and p38 MAP kinase pathways, signs of activated apoptosis and oxidative stress resembling phenotype of premature senescent fibroblasts even though they all came from very young donors. The N2 group showed neither signs of mitochondria response observed in the M group, nor signs of premature senescence observed in the N1 group. No specific functionally meaningful changes, except of signs of activated apoptosis, were detected in the N2 group.

Comparisons with controls, between defined groups and among individual patient cell lines did not show any uniform transcription changes in ATP synthase-related genes explaining pronounced decrease in ATP synthase content and alterations of the other OXPHOS complexes observed at the protein level. The analysis nevertheless confirmed the already known defect in M group and indicated the candidate disease causing genes in N1 and N2 group of patients, and suggested that defects in ATP synthesis lead to deregulation of signal transduction pathways and affect mitochondrial and nuclear DNA replication.

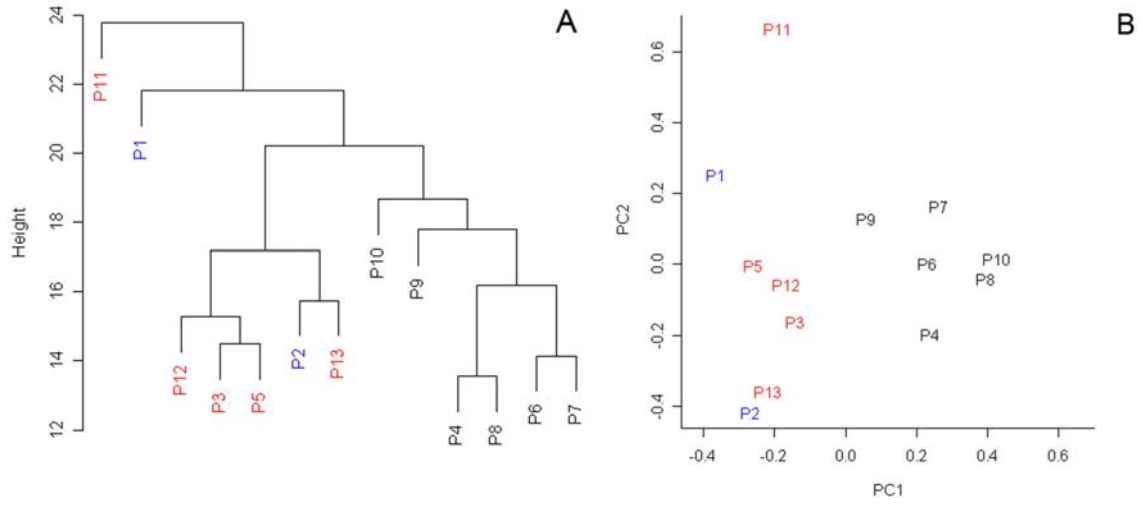

Figure 4: Results of unsupervised clustering methods. A) Dendrogram resulting from two-dimensional hierarchical clustering of all genes across all patient samples performed using Euclidean distance metrics and average linkage clustering algorithm. B) Two-dimensional principal component analysis (PCA) plot of all expression data showing the separation of samples forming N1 group. Patients from M, N1 and N2 groups are shown in blue, black and red, respectively.

2. TMEM70 is a novel factor of ATP synthase biogenesis and its mutations cause isolated enzyme deficiency and neonatal mitochondrial encephalo-cardiomyopathy, Čížková, A., Stránecký, V., Mayr, J. A., Tesařová, M., Havlíčková, V., Paul, J., Ivánek, R., Kuss, A. W., Hansíková, H., Kaplanová, V., Vrbacký, M., Hartmannová, H., Nosková, L., Honzík, T., Drahota, Z., Magner, M., Hejzlarová, K., Sperl, W., Zeman, J., Houštěk, J. and Kmoch, S., *Nature Genetics 2008 Nov;40(11):1288-90.*

To continue our work, we used modern genomics and molecular genetics methods such as whole-genome homozygosity mapping, gene expression analysis and DNA sequencing in individuals with isolated mitochondrial ATP synthase deficiency.

We performed a linkage analysis and homozygosity mapping in eight affected individuals, their healthy siblings and parents from six families. All affected patients shared the region of homozygosity on chromosome 8. To prioritize candidate genes, we intersected the mapping information with gene expression data obtained by the Agilent 44k array. A comparison of patients with healthy individuals illuminated a single gene (*TMEM70)* that has previously been localized in a top-candidate region on chromosome 8, see Fig 5. By sequence analysis of genomic DNA, we identified, in affected individuals, a homozygous substitution, 317-2A>G, located in the splice site of intron 2 of *TMEM70*, which leads to aberrant splicing and loss of *TMEM70* transcript. We carried out the PCR-RFLP analysis, screened for the 317-2A>G mutation among 25 individuals with low ATP synthase content, and found 23 who were homozygous for this mutation

We performed a complementation study of the fibroblasts cell lines of these individuals with wild-type *TMEM70* cloned into the pEF-DEST51 expression vector. We observed restoration of biogenesis and metabolic function of the enzyme complex to a nearly normal level. The phylogenetic analysis revealed TMEM70 homologues in genomes of multicellular eukaryotes and plants, but not in yeast and fungi. TMEM70 is a novel factor of the ATPase biogenesis and appears to be responsible for a vast majority of cases of ATP synthase deficiency of nuclear genetic origin, at least among individuals, particularly Romanies, with mitochondrial energy provision disorders.

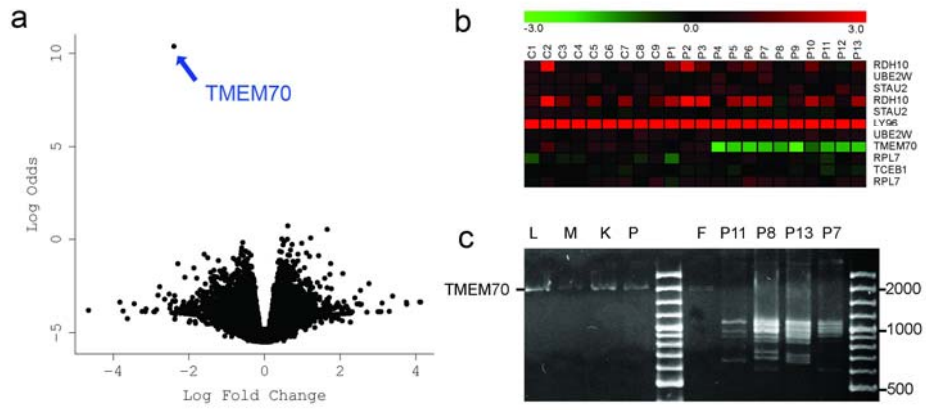

Figure 5: Positional cloning of TMEM70. (a) Gene expression changes between the case and control fibroblast cell lines. The logarithm of the probability that the gene is differentially expressed (log odds) is plotted as a function of the logarithm of the expression ratio (log2 expression ratio) between the case and control samples. (b) Expression matrix of the genes located in the candidate region showing reduced TMEM70 transcript amount in all but one case (P3) with a nuclear defect. Normal TMEM70 transcript amount is present in cases P1 and P2, as these individuals had the mt9205ΔTA microdeletion and not the TMEM70 mutation. The results are shown as log2 ratios of gene expression signal in each sample to that of a common reference sample. (c) TMEM70 cDNA analysis. L, M, K, P and F lanes show presence of a single RT-PCR product in control human liver, muscle, kidney, pancreas and fibroblasts, respectively. Abnormal RT-PCR products were observed in fibroblasts from affected individuals (lanes P11, P8, P13 and P7).

3. HIF and reactive oxygen species regulate oxidative phosphorylation in cancer, Hervouet, E., Čížková, A., Demont, J., Vojtíšková, A., Pecina, P., France-van Hal, N., Keijer, J., Simonnet, H., Ivánek, R., Kmoch, S., Houštěk, J. and Godinot, C., *Carcinogenesis. 2008 Aug;29(8):1528-37.*

This publication resulting from our collaboration with the group of Catherine Godinot from Lyon University, and Jaap Kiejer from the Institute of Food Safety (RIKILT) in Wageningen, was focused on the study of clear cell renal carcinoma (CCRC). This particular type of cancer is caused by inherited mutations in tumor suppressor gene, *VHL* (von Hippel-Lindau factor), which encodes the protein responsible for down-regulation of hypoxia inducible factor (HIF-1α) under normoxia. A decrease in oxidative phosphorylation is characteristic of many cancer types and, in particular, of clear cell renal carcinoma. In the absence of functional VHL protein, HIF1-α and HIF2-α subunits are stabilized and induce the transcription of many genes including those involved in glycolysis and reactive oxygen species (ROS) metabolism.

When *vhl* is transfected in VHL^-, their OXPHOS subunit contents are restored and after inhibition of HIF2-α synthesis by RNA interference in VHL^- the respiratory chain subunit content is also restored. It clearly demonstrated a key role of HIF in OXPHOS

regulation. For better understanding the mechanisms involved in the regulation of respiratory chain subunit amounts by the pVHL/HIF system, we have studied the influence of pVHL on mRNA expression level by a large-scale MWG microarray. We compared gene expression of the 786-0 cell lines (VHL^-) and 786-0 cell lines transfected with *VHL* gene (VHL^+). We didn't observed changes in gene expression neither in OXPHOS subunits and OXPHOS specific assembly factors nor in promitochondrial regulatory genes (PGC1α, NRF1, TFAM). Therefore we concluded that a decreased content of respiratory chain proteins did not came from lower expression of their genes, but probably from the changes in posttranscriptional regulation. Only overexpressed genes were mitochondrial Mn-SOD (manganese superoxide dismutase) and lysyl oxidase, two enzymes producing H_2O_2 and importantly involved in ROS metabolism. The mitochondrial Mn-SOD increase observed in VHL^- cells leads to enhanced conversion of superoxide radicals to molecular oxygen and H_2O_2, which might be a mechanism that the cell developed to survive, see Fig 6.

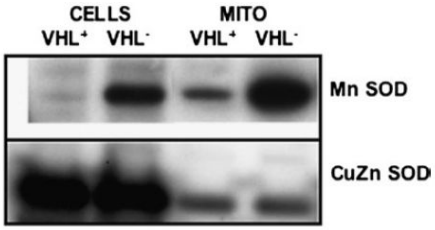

Figure 6: Increased expression of Mn-SOD in 786-0 cells devoid of pVHL. The content of SODs (Mn-SOD and Cu-Zn-SOD) was quantified by Western blot. For analysis, 15 μg of protein from cell extracts or 5 μg of mitochondrial protein were loaded.

4. Mutations in TMEM76 cause acetyl coenzyme A: α-glucosaminide *N*-acetyltransferase deficiency in mucopolysaccharidosis type IIIC (Sanfilippo C) patients,

Hřebíček, M., Mrázová, L., Majewski, J., Durand, S., Seyrantepe, V., Roslin, N. M., Nosková, L., Hartmanová, H., Ivánek, R., Čížková, A., Poupětová, H., Sikora, J., Uřinovská, J., Stránecký, V., Zeman, J., Verner, A., Beesley, C. E., Maire, I., Poorthuis, B., van de Kamp, J., van Diggelen, O., Hudson, T. J., Fujiwara, T. M., Morgan, K., Kmoch, S. and Pshezhetsky, A. V., *The American Journal of Human Genetics, 2006 Nov;79(5):807-19.*

The last article deals with identification of gene causing *N*-acetyltransferase deficiency which manifested as mucopolysacharidosis type IIIC. This research was done in collaboration with the group of Dr. Pshezhetsky from Université de Montreal. Mucopolysaccharidosis IIIC (MPS IIIC, or Sanfilippo C syndrome) is an autosomal recessive lysosomal storage disorder caused by deficiency of the lysosomal membrane enzyme acetyl–CoA:α-glucosaminide *N*-acetyltransferase (*N*-acetyltransferase), which leads to impaired degradation of heparan

sulphate. For identification of molecular basis of this disorder, we used a number of approaches such as purification of human and mouse lysosomal *N*-acetyltransferase, linkage analysis and gene expression analysis. We report the narrowing of the candidate region by genotyping for 22 microsatellite markers to a 2.6-cM interval between *D8S1051* and *D8S1831*. For all of 32 genes located in the candidate interval, a custom oligonucleotide-based microarray assay was designed. For each gene, single oligonucleotide were designed and spotted in quadruplicate on the array. Transcript levels were compared in two patients with MPS IIIC and four healthy controls and only *TMEM76* (transmembrane protein 76 gene) showed a significant reduction of transcript level. *TMEM76*, now termed *HGSNAT* (heparin acetyl–CoA: α-glucosaminide *N*-acetyltransferase), is the gene that causes MPS IIIC when mutated, see Fig 7. This gene encodes a 73 kDa protein with predicted multiple transmembrane domains and glycosylation sites.

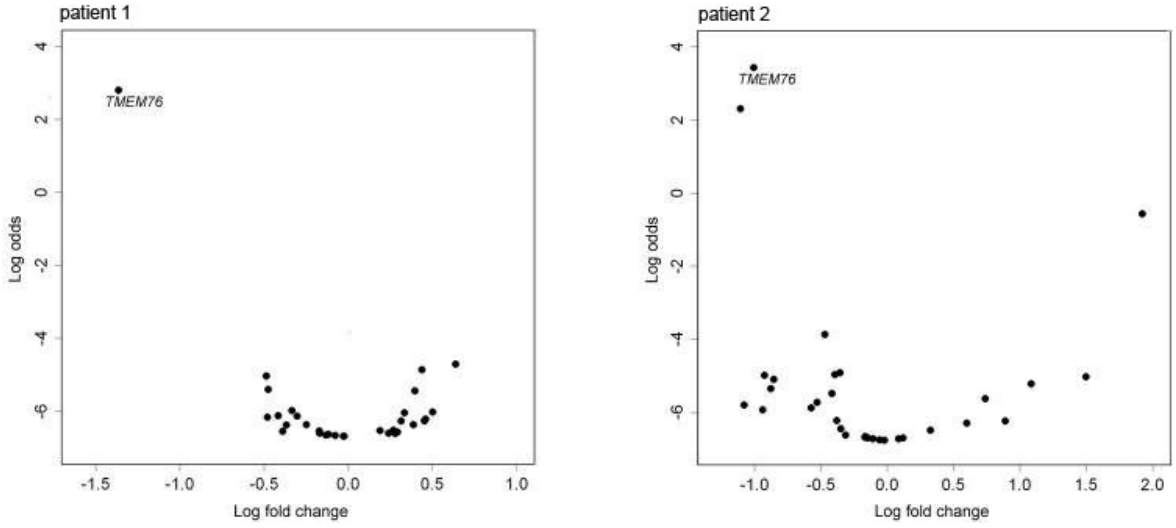

Figure 7: Volcano plot of genes located within the MPS IIIC candidate region, showing significantly reduced expression of the *TMEM76* gene in white blood cells of two patients with MPS IIIC. The natural logarithm of the probability that the gene is differentially expressed (Log odds) is plotted as a function of the logarithm of the gene-expression log2 fold change (Log fold change) between the patient and control samples.

Contribution of dissertant to published articles

Presented results were achieved in a team effort and the dissertant carried out construction, validation and testing of oligonucleotide microarray (h-MitoArray), sample collection, nucleic acid isolation and quality control measurement, fluorescent labeling, gene expression analysis using different platform (h-MitoArray, Agilent, MWG), pathway analysis and data interpretation, sequencing, transcript analysis, cell culturing, transfections and TMEM70 complementation studies.

4 CONCLUSIONS

The results of the thesis clearly demonstrate the benefit of new genomics approaches for characterization of inherited metabolic disorders and identification of disease causing genes. Specifically the following results have been achieved:

A) Gene expression profiling using different platforms

- We developed an oligonucleotide microarray (h-MitoArray) allowing the expression analysis of 1632 human genes involved in mitochondrial metabolism
- We carried out a pilot hybridization experiment which validated oligonucleotide probes design, control feature and reproducibility of this platform and highly correlated with commercial platform Agilent

B) Gene expression analysis in unique group of patients with ATP synthase deficiency

- We compared 9 controls and 13 patients with mitochondrial F_1F_o ATP synthase deficiency by expression profiling, functional annotation and pathway analyses of differentially expressed genes which defined three subgroups of patient cell lines - M, N1 and N2 group
- We identified, using positional cloning, the disease causing gene *TMEM70*
- We identified a homozygous substitution, 317-2A>G, located in splice site of intron 2 of *TMEM70* in 23 patients, primarily from the Roma population
- We discovered TMEM70 as a novel ancillary factor in biogenesis of mitochondrial ATP synthase in higher eukaryotes

C) Downregulation of mitochondrial biogenesis in renal carcinoma cells

- We observed a dramatic over-expression in mitochondrial Mn-SOD and lysyl oxidase, both involved in ROS metabolism. It implicates that ROS might play an important regulation role in the metabolism of renal carcinoma cells

D) Disease causing gene characterization

- We developed and prepared a custom oligonucleotide-based microarray assay designed for 32 genes located in the candidate interval and we identified the gene *HGSNAT (TMEM76),* responsible for Mucopolysaccharidosis IIIC
- We have established the platform relevant for diagnosis and identification of disease causing genes of metabolic disorders, which is now used in various projects, e. g. in expression and pathway analysis in COX deficiency due to *SURF1* mutation and ATP synthase deficiency due to mutation in mtDNA gene *ATP6* (NARP mutation)

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6 LIST OF PUBLICATIONS

Čížková, A., Stránecký, V., Mayr, J. A., Tesařová, M., Havlíčková, V., Paul, J., Ivánek, R., Kuss,A. W., Hansíková, H., Kaplanová, V., Vrbacký, M., Hartmannová, H., Nosková, L., Honzík, T., Drahota, Z., Magner, M., Hejzlarová, K., Sperl, W., Zeman, J., Houštěk, J. and Kmoch, S. Nature Genetics 2008. IF (2008) = 30.259
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Čížková, A., Stránecký V., Ivánek, R., Hartmannová, H., Nosková, L., Piherová, L., Tesařová, M., Hansíková, H., Honzík, T., Zeman, J., Divina, P., Potocká, A., Paul, J., Sperl, W., Mayr, J., Seneca, S., Houštěk, J. and Kmoch, S., BMC Genomics. 2008 Jan 25;9:38. IF (2008) = 3.926
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Hervouet, E., **Čížková, A.**, Demont, J., Vojtíšková, A., Pecina, P., France-van Hal, N., Keijer, J., Simonnet, H., Ivánek, R., Kmoch, S., Houštěk, J. and Godinot, C., Carcinogenesis. 2008 Aug;29(8):1528-37. IF (2008) = 4.930
HIF and reactive oxygen species regulate oxidative phosphorylation in cancer.

Hřebíček, M., Mrázová, L., Majewski, J., Durand, S., Seyrantepe, V., Roslin, N. M., Nosková, L., Hartmanová, H., Ivánek, R., **Čížková, A.**, Poupětová, H., Sikora, J., Uřinovská, J., Stránecký, V., Zeman, J., Verner, A., Beesley, C. E., Maire, I., Poorthuis, B., van de Kamp, J., van Diggelen, O., Hudson, T. J., Fujiwara, T. M., Morgan, K., Kmoch, S. and Pshezhetsky, A. V., The American Journal of Human Genetics 2006 Nov;79(5):807-19. IF (2006) = 12.629
Mutations in TMEM76 Cause Mucopolysaccharidosis IIIC (Sanfilippo C Syndrome).

Čížková A., *Genové čipy – perspektivní nástroj molekulární biologie i moderní medicíny*, ŽIVA 03/2005

7 AWARDS, GRANTS

Awards:

2008, 18.11. Česká hlava, Kategorie Doctorandus, cena Vítkovic a.s.

2009, 26.2. Bolzanova cena, Kategorie lékařských věd

Grants - principal investigator:

2005 – 2006 GAUK 54/203208 27/05 Study of nucleomitochondrial interactions using DNA microarray

2007 – 2008 GAUK 257467 46707 Gene expression study of patients with mitochondrial ATP synthase defect

2009 – 2010 GAUK 259089 56209 Function of TMEM70 in mitochondrial biogenesis and metabolism