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PhD thesis – short report

APPLICATION OF NOVEL GENOMIC TECHNIQUES IN STUDIES OF PATHOGENESIS OF SELECTED RARE INHERITED DISORDERS

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SUMMARY

Rare diseases are a heterogeneous group of disorders. Knowledge of their molecular basis is poor and till recently there were no appropriate methodical approaches due to a limited number of patients. Novel genomic techniques, especially the DNA array technology and the next generation sequencing emerging in last few years, enabled studies of these diseases even in small families and sporadic cases.

This PhD thesis focuses on application of novel genomic techniques in studies of rare inherited diseases. It describes a use of DNA array technology in linkage analysis, analysis of differential gene expression, analysis of copy number variations and homozygous mapping, and a use of next generation sequencing technology. Combination of these methods was used for identification of molecular basis of adult neuronal ceroid lipofuscinosis, Rotor syndrome, isolated defect of ATP synthase and mucopolysaccharidosis type IIIC.

SOUHRN

Vzácná onemocnění jsou heterogenní skupinou onemocnění, jejichž molekulární podstata je často neznámá. Pro jejich studium nebyly donedávna z důvodů malého počtu pacientů k dispozici vyhovující metodické postupy. Díky novým přístupům ke studiu genomu, především díky technologiím DNA čipů a s rozmachem sekvenování nové generace, je v současné době možno studovat vzácná onemocnění i u jednotlivých rodin nebo u sporadických případů.

Tato dizertační práce se zabývá aplikací nových genomických technik při studiu vzácných dědičných chorob. Popisuje využití technologie DNA čipů pro účely vazebné analýzy, analýzy změn genové exprese, analýzy změn počtu kopií a homozygotního mapování a dále využití sekvenování nové generace. Kombinace těchto metodických postupů byly použity pro studium molekulární podstaty adultní formy neuronální ceroidní lipofuscinózy, Rotorova syndromu, izolovaného defektu ATP syntázy a mukopolysacharidózy typu IIIC.

INTRODUCTION

The PhD thesis focuses on application of novel genomic techniques – DNA microarrays and next generation sequencing – for studies of several rare inherited disorders. These techniques have been widely available for last five to seven years and enabled time- and cost-effective identification of disease-causing genes. At the Institute of Inherited Metabolic Disorders we have been following the rapid development of these methodological approaches and have been using a large spectrum of DNA arrays starting with "home-made" custom arrays, then replacing them by commercially available ones, and eventually switching to the whole-exome next-generation sequencing.

In the PhD thesis I intend to present usefulness and capabilities of these methods using examples of several rare inherited mitochondrial, neuropsychiatric, hepatologic and lysosomal storage diseases and highlight their variable use depending on the type of inheritance and accessibility of material.

Rare diseases

Rare diseases are defined as diseases with a prevalence lower than 1:2000 in the European Union or 1:1250 in USA.[1] According to the World Health Organization there are more than 5000 disorders that fit into this definition. It is estimated that there is about 30 million of patients in Europe and 25 million patients in North America.[2] Rare diseases form a heterogeneous group of disorders affecting different organs and presenting by various clinical features. They result in reduced quality of life of patients and their families, and in a loss of social contacts and economic opportunities. Knowledge of the molecular basis of majority of the rare diseases is poor. Studies of rare diseases were recently limited both by limited investments as well as by methodological obstacles.

Research on rare diseases is justifiable in many respects. At the socio-political, cultural and ethical level there is the patients` right to profit from scientific progress, right to correct diagnosis and to first-class medical treatment. At the level of basic research, identification of disease-causing genes may contribute to better understanding of biological processes. As the most of rare diseases are monogenic, patients with given defect may serve as unique models for understanding of physiological and patophysiological processes. Definition of causal genes may further contribute to studies of complex diseases and to broader understanding of metabolic, signal and regulatory pathways. This was the case of Alzheimer`s disease pathogenesis based on characterization of mutations in *APP*, *PSEN1* and *PSEN2* genes in familiar forms of Alzheimer`s disease.[3] Identification of disease causing genes further allows description of the effects of different mutations on the phenotypical and clinical variability, and enables to delineate genetic factors and modifying genes. The disease-gene identification can help in search of a specific treatment by means of influencing specific pathways.

Studies of rare diseases contribute to growing amount of information about elementary biological processes. The focus on rare diseases may thus bring correct diagnosis, targeted treatment and prevention not only for rare diseases but also for many others.

Methodological approaches to studies of rare diseases

In the last decade technological advances broadened substantially possibilities to study molecular mechanisms and cellular processes. New technologies, such as the DNA array technology and whole-exome sequencing enable us to study not only isolated events but describe the processes in their complexity, i.e., on the scale of a whole genome, exome, transcriptome, metabolome, etc.

DNA array technology

DNA microarrays allow simultaneous detection of thousands and tens of thousands of DNA fragments. The main principle is a hybridization of fluorescently labelled DNA fragments to probes immobilized to the surface of the array and consequent analysis of the fluorescence signal. The DNA array technology emerged in the last decade of twentieth century and has gone through a rapid expansion with the release of human genome sequence by the Human Genome Sequencing Consortium.[4,5]

Microarrays can be manufactured in different ways. In the *in-situ* synthesis, single bases are sequentially immobilized on the microarray surface using light deprotection of functional groups. This happens with the help of sets of masks (Affymetrix), sets of micromirrors (Roche Nimblegen), or by a non-contact inkjet printing process (Agilent Technologies). Another form of microarray production is immobilization of probes on silica beads (Illumina). Both the methods enable production of high-density arrays containing up to millions of probes which can be used for studies at the whole-genome level.Furthermore, at the outset of the DNA microarray technology history, also so called spotted arrays played an important role in many laboratories. These arrays are produced by depositing (spotting) individual probes at the surface of a microscopic slide using robotic systems. This approach allowed cost-effective fabrication of custom DNA arrays. At the Institute of Inherited Metabolic Disorders, we produced several custom arrays and optimized methods of probe immobilization, fluorescent labelling, image analysis and statistical analysis. We used these arrays in studies of differential gene expression in patients with isolated the ATP synthase deficiency (project h-MitoArray)[6,7] and in patients with the lysosomal storage disease mucopolysaccharidosis type IIIC [8]. Also, a custom array for comparative genomic hybridization was produced to study the molecular basis of Rotor syndrome [9].

There are many types and uses of DNA arrays. They are widely used for genotyping, gene expression analysis, comparative genomic hybridization (CGH), and copy-number variant (CNV) analysis. The commercial vendors offer DNA arrays for chromatin immunoprecipitation, epigenetic studies, miRNA analysis, promotor analysis, alternative splicing analysis, and protein arrays.

Focusing on the rare diseases, genotyping arrays are commonly used for linkage analysis in families. They allow identification of candidate regions containing potential disease causing gene(s), reveal presence of small insertions or deletions and define homozygous regions. Copy number variation analysis by high density genotyping arrays or by CGH arrays results in characterization of nonpolymorfic structural changes such as large deletions, insertions, tandem or segmental duplications, inversions or translocations. Use of expression arrays may be helpful for prioritization of candidate genes in delimited candidate regions.

Next-generation sequencing

Next generation sequencing methods are high-throughput technologies enabling parallelization of the sequencing process and producing thousands or millions of sequences at once. These methods may be extremely useful for studies of rare diseases whose genetic basis failed to be identified by standard approaches. Recently next generation sequencing helped in identification of disease causing genes in Kabuki syndrome [10], Fowler syndrome [11] or Charcot-Marie-Tooth disease [12].

Although whole-genome sequencing is available in principle, most of the projects are limited in terms of throughput, cost efficiency and capabilities of bioinformatic analysis. Whole-exome sequencing is mainly preferable for detecting genomic variants in protein-coding and RNA genes by using various capturing approaches. Exome sequencing has rapidly become one of the main tools for studying the genetic cause of Mendelian diseases.

Sequencing technologies include a number of methods such as template preparation, exome or custom capture, sequencing, imaging and data analysis.

Template preparation generally involve randomly breaking of genomic DNA into smaller fragments, ligation of specific adaptors, amplification (not needed in single molecule sequencing approach) and fragment size selection. Bar-coding of individual libraries then enables pooling of samples and increases cost- and labour-effectiveness of library preparation.

Target-enrichment strategies involve selective capturing of specific DNA sequences before sequencing. There are several enrichment methods, each with unique advantages and disadvantages. In the multiplex PCR-based approach, multiple primer pairs are used simultaneously in one reaction (RainDance Technologies). Molecular inversion probes are designed with universal spacer region flanked by target-specific sequences. These probes anneal to the target regions, DNA polymerase and a ligase fill the gap, and targeted DNA is PCR-amplified. In the hybrid capture-based approach, adaptor-modified DNA libraries are hybridized to target-specific probes either immobilized on microarrays or in solution (Agilent Technologies, Roche Nimblegen, Illumina).[13,14] Exome and custom capture systems are available commercially. The main limitation of all these methods is an incomplete coverage of the whole exome and incomplete information about all exons and genes in databases.

Targeted DNA fragments are either directly sequenced (Helicos Biosciences, Pacific Biosciences), or further amplified by emulsion PCR (Roche Nimblegen, Life Technologies, Polonator) or amplified on solid-phase (Illumina).

The sequencing strategy depends on the selected method of library preparation, amplification and immobilization. Cyclic reversible termination (Fig. 1A) uses reversible terminators enabling incorporation of just one fluorescently modified nucleotide (Illumina, Helicos Biosciences, LaserGen, Pacific Biosciences). Sequencing by ligation (Fig. 1B) uses hybridization of fluorescently labelled probe, ligation to the primer or the previous probe and fluorescence imaging according to specific code (two-base-encoded probes) (Life Technologies). By pyrosequencing (Fig. 1C), incorporation of dNTP to DNA template is detected by bioluminescence (Roche 454 sequencing system).

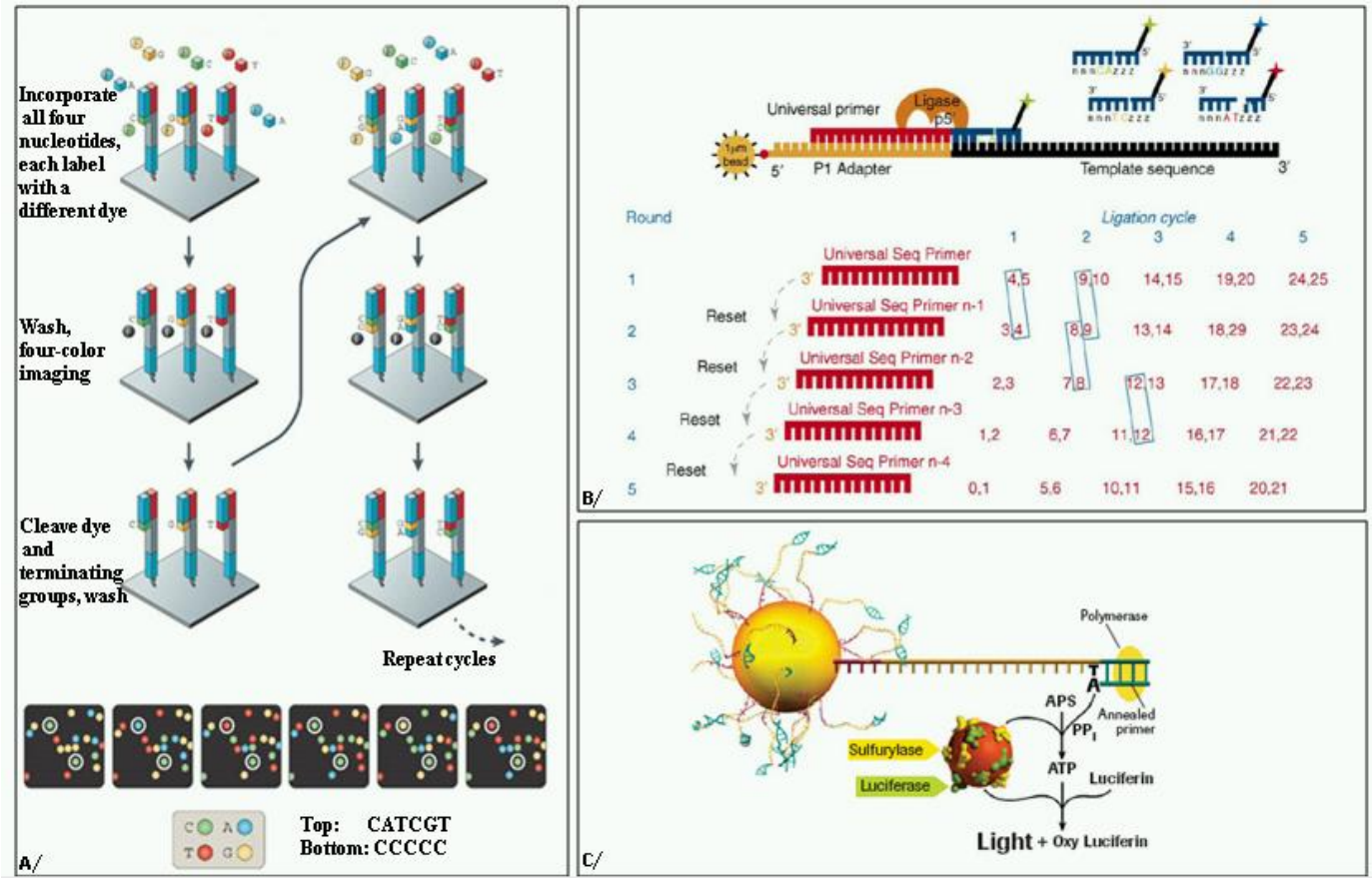

Fig. 1 Individual sequencing strategies.
A/ Cyclic reversible termination, modified according to [15]
B/ Sequencing by ligation (http://www.invitrogen.com)
C/Pyrosequencing (http://454.com/)

Many more sequencing methods are under development or already in practice. Ion Torrent Systems Inc. (Life Technologies) developed a Personal Genome Machine, a system based on the detection of hydrogen ions released during DNA polymerisation. These instruments are rapidly expanding to clinical practice. Other developed methods are based on fluorescent resonance energy transfer, on nanopore sequencing or single molecule real-time sequencing. [16]

Bioinformatic analysis of the next generation sequencing data is a challenging and rapidly developing field. Primary data has to be transformed into short sequences, which are further aligned to a reference genome. Individual variations are detected subsequently. Many commercial as well as free types of software may be used for each step of analysis and their proper selection is a tough task for experienced bioinformatician.

List of annotated variants is further filtered by using information from available databases (dbSNP, 100 Genomes, HapMap project, Exome Variant Server, in-house databases). Candidate variants must be in concordance with the type of inheritance and must segregate with phenotype. Software prediction of pathogenicity of individual changes (SIFT, Polyphen, SNAP) may be helpful as well as conservation score. Previous knowledge about the individual gene and protein and broader biological context may give a clue about relevance of candidate variation in context of studied disease.

AIMS OF THE THESIS

The main goal of the PhD thesis has been an application of novel genomic techniques in studies of selected rare inherited disorders. These techniques are suitable for identification of candidate genes and genomic changes; their causality has to be further proven by a set of molecular-genetic, biochemical and cell biology methods. Use of these consecutive methods is therefore an inseparable part of the thesis.

The main aims of the thesis are:

1. Study of autosomal dominant adult neuronal ceroid lipofuscinosis using combination of linkage analysis, differential expression analysis, copy number variation analyses and exome sequencing.
2. Study of Rotor syndrome using homozygous mapping and copy number variation analyses.
3. Study of autosomal recessive isolated deficiency of ATP synthase using homozygous mapping and genotyping.
4. Study of mucopolysaccharidosis type IIIC (MPSIIIC) using differential gene expression analysis of genes delimited by previous linkage analysis.

SUMMARY OF THE RESULTS

Study of molecular basis of autosomal dominant neuropsychiatric disorders.

a/ Elucidation of molecular basis of autosomal dominant adult neuronal ceroid lipofuscinosis (Kufs disease).

The neuronal ceroid lipofuscinoses (NCLs) are a heterogeneous group of inherited neurodegenerative disorders with an incidence of between 1 and 30 per 100,000. Common findings in the NCLs are an accumulation of autofluorescent storage material in neural and peripheral tissues and neurodegeneration. Although more than 450 mutations in nine genes—*CLN1 (PPT1), CLN2 (TPP1), CLN3, CLN5, CLN6, CLN7 (MFSD8), CLN8, CLN10 (CTSD) CLN11 (KCTD7, GRN), CLN12 (ATP13A2)* and *CLN13 (CTSF*)—have been identified, the genetic and molecular basis of adult-onset NCL with dominant inheritance (Parry type [MIM162350]) remained unknown.

Autosomal-dominant adult-onset neuronal ceroid lipofuscinosis (ANCL, Kufs disease, Parry type of Kufs disease) was first described in a family of British descent from New Jersey, USA (Parry disease)[17] and in a second family reported in Spain [18]. More recently, a large American family with English ancestry [19], another family from Alabama, USA [20] and a third family from the Netherlands [21] were presented. Common characteristics of affected individuals included generalized seizures, movement disorders, cognitive deterioration, and progressive dementia; the age of onset varied between 25 and 46 years.

At the Institute of Inherited Metabolic Disorders, we have described a Czech family with autosomal-dominant ANCL in whom, by using a combination of linkage mapping, gene-expression analysis, and exome sequencing, we have identified a unique heterozygous mutation c.346_348delCTC (p. Leu116del) in *DNAJC5* encoding cysteine-string protein alpha (CSPα [MIM 611203]). The same or a second heterozygous *DNAJC5* mutation (c.344T>G (p. Leu115Arg)) was found in four out of twenty additional unrelated ANCL families and/or simplex cases. Haplotype analyses proved that both mutations appeared independently in different lineages. Identified mutations were absent in 200 control samples of European descent and were not present in the dbSNP or 1000 Genomes databases.

CSPα is a highly conserved membrane protein localized in synaptic vesicles of neuronal cells. It associates with Hsc70 and SGT proteins and forms an enzymatically active chaperone complex.[22] In cooperation with other chaperones it ensures correct conformation of many proteins essential for the functionality of synapses.[23] CSPα deletion causes progressive neurodegeneration and reduced life span in *Drosophila melanogaster* (ZINSMAIER et al. 1994) and in knockout mice.[24,25]

Both mutations (p.Leu116del and p.Leu115Arg) affect conserved dileucine residues located in the cysteine-string domain implicated in palmitoylation and membrane trafficking of CSPα.[26] Using functional studies in transfected cell lines, we have proved that these mutations affect palmitoylation and intracellular localization of CSPα. The analysis of *post-*

mortem brain specimens showed decrease or absence of CSPα protein in patients` samples and revealed also presence of insoluble CSPα-containing aggregates after chemical depalmitoylation. The resulting depletion of functional CSPα may cause presynaptic dysfunction and the progressive neurodegeneration observed in affected individuals. Dysfunction of chaperone complex might affect the folding quality of many client proteins and make them vulnerable to aggregation and degradation.[27]

Our finding of neurodegenerative disease caused by mutation in *DNAJC5* thus confirms a neuroprotective role for CSPα in humans and advocates detailed investigation of CSPα in the NCLs and other neurodegenerative diseases presenting with neuronal protein aggregation. Our work also represents a major step in the genetic dissection of a genetically heterogeneous group of ANCLs, as we have identified causal mutation in about 25% of known cases.

The article „Mutations in *DNAJC5*, Encoding Cysteine-String Protein Alpha, Cause Autosomal-Dominant Adult-Onset Neuronal Ceroid Lipofuscinosis." was published in 2011 in The American Journal of Human Genetics (IF = 11,680). Publication of the results was followed by subsequent articles describing the same mutations in same or another families or sporadic cases.[28,29]

b/ Identification of presenilin 1 mutation in a family included into the set of suspect cases with Kufs disease.

By identification of mutations in *DNAJC5* we have elucidated molecular basis of about 25% of known cases of adult onset neuronal ceroid lipofuscinosis. We decided to use similar approach in study of another family diagnosed by autosomal dominant Kufs disease.

By using a combination of linkage mapping, copy number variation analysis and exome sequencing we obtained 7 potentially disease-causing variants, of which the heterozygous mutation c.509C>T (p.Ser170Phe) in presenilin 1 (*PSEN1*) emerged as causative. Mutations in *PSEN1*, presenilin 2 (*PSEN2*) and Aβ amyloid precursor protein (*APP*) are known to be responsible for a large number of familiar early onset Alzheimer disease.[3] c.509C>T (p.Ser170Phe) mutation in *PSEN1* is present in the dbSNP database with accession number rs63750577 and is referred to as pathogenic. It is acknowledged as a disease-associated mutation in the Alzheimer Disease & Frontotemporal Dementia Mutation Database (http://www.molgen.ua.ac.be). Our sequence analysis of the mutation-bearing fragment showed its segregation with the phenotype in the family.

Identical mutation p.Ser170Phe has already been reported in the literature in other family[30] and three sporadic cases[31-33] until to date. Phenotype comparison revealed that in two reported cases the motor symptoms encompassed a prominent cerebellar phenotype early in the disease which is comparable to our cases. *PSEN1* is a membrane protein that is part of the γ-secretase complex.[34] To date 185 mutations in *PSEN1* have been described leading to autosomal dominant AD.

The original diagnosis was assessed as autosomal dominant Kufs disease since some features in our patients were reminiscent of adult onset NCL To further identify potentially

disease-modifying variants we functionally annotated the candidate variants identified by exome sequencing. This analysis revealed a heterozygous mutation c.C173>T (p.A58V) in cathepsin D (*CTSD*), and sequence analysis showed that all three affected individuals of the last generation bearing *PSEN1* p.Ser170Phe mutation are heterozygous for this mutation, which they inherited from their unaffected father. *CTSD* is likely involved in AβPP processing[35] and recessive mutations of *CTSD* cause NCL10[36]. In addition, the role of *CTSD* variants has been studied in AD[37], and specifically the heterozygous *CTSD* variant p.A58V has been associated with an increased risk for AD[38]. The presence of the p.A58V variant may thus have an additional impact on the extremely early onset and course of disease in our *PSEN1* Ser170Phe family.

In conclusion, this work did not reveal another disease causing gene for ANCL, as the diagnosis of early onset AD was missed. Nevertheless, our work demonstrates effectiveness of genome-wide linkage analysis and exome sequencing based genetic testing in single families.

The article „Cerebellar dysfunction in a family harbouring the PSEN1 mutation co-segregating with a Cathepsin D variant p.A58V.“ was accepted for publication in 2013 in Journal of the Neurological Sciences (IF = 2,353).

Study of molecular basis of Rotor syndrome.

Rotor syndrome (RS, MIM237450) is a rare, benign hereditary conjugated hyperbilirubinemia. It is coupled with coproporphyrinuria and with strongly reduced liver uptake of many diagnostic compounds. RS is an autosomal recessive disorder that clinically resembles another conjugated hyperbilirubinemia – the Dubin-Johnson syndrome (DJS, MIM 237500). Unlike patients with DJS, patients with RS lack the typical hepatocyte pigment deposits. Total urinary excretion of copropophyrins is greatly increased in RS.[39] In RS there is delayed plasma clearance of unconjugated anionic dyes and neither the liver nor the biliary tree is visualized by cholescintigraphy.[40,41] The molecular basis of RS was unknown.

Rotor syndrome was studied at the Institute of Inherited Metabolic Disorders in cooperation with the Institute for Clinical and Experimental Medicine. As the features of RS and DJS partly overlap, we hypothesized that RS and DJS may be allelic variants of the same disorder. Mutation and histological analyses of *ABCC2* gene which is known to cause DJS [42,43] were performed, but no pathogenic mutations in two affected subjects with RS were found and normal localization of *ABCC2* protein was confirmed. As the Sanger sequencing does not allow to detect large insertions or deletions, we designed, manufactured and optimized custom array for copy number changes caused by exon deletions or duplications of several selected genes including *ABCC2* using in-house robotic spotter. Comparative genomic hybridization revealed no significant copy number changes in any of 32 exons of *ABCC2* in two studied patients. This finding supported mutation and immunohistochemical analyses. It was shown that RS is not an allelic variant of DJS. Article „Rotor-type hyperbilirubinaemia has no defect in the canalicular bilirubin export pump.“ was published in 2007 in Liver International (IF = 2, 559).

We further continued to study molecular basis of RS and genotyped 8 available RS families. We performed homozygosity mapping and found single homozygous region on chromosome 12. Three distinct homozygous haplotypes (R1-R3) segregated with the RS. Intersection of these haplotypes defined a candidate region including five genes. A parallel genome-wide copy number analysis detected a homozygous deletion within the *SLCO1B3* gene in the R1 haplotype and a large homozygous deletion encompassing *SLCO1B3*, *SLCO1B1* and LST-3TM12 genes. Sequence analysis revealed pathogenic mutations affecting both *SLCO1B1* and *SLCO1B3* genes in each of the haplotypes (exon deletion, nonsense mutations and splice mutations). The mutations showed consistent autosomal recessive segregation with the RS phenotype in the investigated families. All of these mutations would severely disrupt or annihilate proper protein expression and function, the severity of the mutations was independently supported by immunohistochemical studies of corresponding proteins OATP1B1 and OATP1B3. Only complete deficiency of both alleles of *SLCO1B1* and *SLCO1B3* results in RS, a single functional allele can prevent the symptoms. RS is thus a two-gene disorder caused by a complete deficiency of OATP1B1 and OATP1B3. OATP1B1 and OATP1B3 are organic anion transporting polypeptides mediating cellular uptake of highly diverse compounds including bilirubin glucuronide, bile acids, steroid and thyroid hormones, drugs, toxins and their conjugates.[44,45] OATP1B1 polymorphisms have previously been linked to drug hypersensitivities.[46]

In parallel, mice models deficient in Oatp1a/1b and Abcc3 proteins were studied at The Netherland Cancer Institute. It was shown that Abcc3 secretes bilirubin conjugates into the blood, while Oatp1a/1b transporters mediate their hepatic reuptake. Transgenic expression of human OATP1B1 or OATP1B3 restored the function of detoxification-enhancing liver-blood shuttle in Oatp1a/1b- deficient mice. Within liver lobules, this shuttle may allow flexible transfer of bilirubin conjugates formed in upstream hepatocytes to downstream hepatocytes, thereby preventing local saturation of further detoxification processes and hepatocyte toxic injury.

These two independent studies explained the genetic and molecular basis of RS and demonstrated an Abcc3-, OATP1B1- and OATP1B3-driven liver-blood shuttling loop in mice and most likely also in humans. This study changes the view at the bilirubin excretion pathway and opens possibilities of studies of drug hypersensitivities.

The article „Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver.“ was published in 2011 in The Journal of Clinical Investigations (IF = 14,152).

Study of molecular basis of isolated defect of ATP synthase.

Mitochondrial diseases are a heterogeneous group of disorders resulting from various defects of mitochondrial biogenesis and maintenance, respiratory chain complexes or individual mitochondrial proteins. The prevalence is estimated as at least 1:5000.[47] The general feature is an energy metabolism defect related to decrease of ATP production. The diseases may be caused by mtDNA mutations or result from mutations in genes encoded in

nuclear DNA. The majority of the nuclear encoded diseases are inherited as an autosomal recessive traits and produce severe and usually fatal phenotypes in infants.[48]

At the Institute of Inherited Metabolic Disorders in collaboration with the Institute of Physiology of the Academy of Sciences we focused on a set of patients with isolated defect of ATP synthase. ATP synthase deficiencies belong to the most severe mitochondrial disorders presenting with neonatal lactic acidosis, encephalocardiomyopathy and/or variable central nervous systems involvement and 3-methylglutaconic aciduria.[49,50]

At the beginning of this project no complete coverage of mitochondrial tRNA, rRNA and OXPHOS structural subunits have been available on custom arrays. Therefore, we designed, produced and validated an oligonucleotide microarray focused on gene expression profiling of human mitochondria related genes (h-MitoArray) and searched for gene expression changes in genetically heterogeneous group of 13 patients with ATP synthase deficiency. We compared the expression profiles and carried out functional annotation, gene enrichment and pathway analyses. The analysis defined three subgroups of patient cell lines. First subgroup contained patients with known mtDNA mutation, their transcription profile pointed to synchronized suppression of mitochondrial biogenesis and G1/S arrest. Second subgroup showed signs of activated apoptosis and oxidative stress resembling phenotype of premature senescent fibroblasts. No common specific changes were detected in third subgroup. Evaluation of individual gene expression profiles confirmed already known mtDNA defects and indicated several candidate disease causing genes for nuclear defects.

Article „Development of a human mitochondrial oligonucleotide microarray (h-MitoArray) and gene expression analysis of fibroblast cell lines from 13 patients with isolated F(I)F(o) ATP synthase deficiency. “ was published in 2008 in BMC Genomics (IF = 3, 926).

To further identify the genetic defect in individuals with isolated deficiency of ATP synthase we genotyped eight affected individuals, their healthy siblings and parents from six families of Roma origin and performed linkage analysis and homozygosity mapping. To prioritize candidate genes, we intersected the mapping information with data from previously performed gene expression analysis on Agilent 44K array. This analysis illuminated a single gene, *TMEM70*, as it has previously been localized in homozygous region on chromosome 8, showed reduced transcript amount in fibroblast cell lines from affected individuals and encoded protein characterized as a mitochondrial one. Through sequence analysis of genomic DNA we found a homozygous substitution c.317 -2A>G, located in splice site of intron 2 of *TMEM70*, which leads to aberrant splicing and loss of *TMEM70* transcript. We proved autosomal recessive segregation of the mutation in families and found the same homozygous mutation in 23 out of 25 patients with low ATP synthase content available at the institution. *TMEM70* complementation experiments showed full restoration of ATP synthase function. Using phylogenetic analysis, we found *TMEM70* homologs in multicellular eukaryotes and plants, but not in yeast and fungi.

We have identified *TMEM70* as a protein involved in the biogenesis of the ATP synthase in higher eukaryotes and shown that its defect is relatively frequent among individuals with mitochondrial energy provision disorders.

The article „*TMEM70* mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalocardiomyopathy.“ was published in 2008 in Nature Genetics(IF = 30, 259).

Study of molecular basis of mucopolysaccharidosis type IIIC.

Mucopolysaccharidosis IIIC (MPSIIIC, Sanfilippo syndrome C, MIM 252930) is a rare autosomal recessive lysosomal storage disorder caused by the deficiency of acetyl-coenzyme A: α-glucosaminide N-acetyltransferase. It leads to impaired degradation of heparan sulphate, polysaccharide found in proteoglycans associated with the cell membrane. Patients manifest symptoms during childhood with progressive and severe neurological deterioration. Major symptoms are hyperactivity, sleep disorders, loss of speech, behavioural abnormalities, neuropsychiatric problems, mental retardation, hearing loss, and relatively minor visceral manifestations.[51,52] Disease causing gene was not known, linkage analyses delineated candidate 8,3 cM interval on chromosome 8.[53]

At the Institute of Inherited Metabolic Disorders we studied five patients from four non related families and performed linkage analysis in every family. In parallel, genotyping of 22 microsatellite markers in 60 patients and 44 unaffected relatives was performed in collaborating institution in Montreal. This lead to narrowing of candidate region to 2,6 cM interval containing 32 known and predicted genes. To compare the level of transcripts of these 32 genes between normal control cells and those from patients with MPS IIIC, we designed and produced an oligonucleotide microarray. The analyses showed a statistically significant reduction of the transcript level of *TMEM76* gene in the cells of two patients with MPSIIIC. This gene was also chosen as a candidate gene by bioinformatic search on the basis of biochemical characteristics of the protein encoded by *TMEM76*. DNA mutation analysis identified 27 *TMEM76* mutations in the DNA of 30 MPSIIIC-affected families. Functional importance of *TMEM76* gene in the pathogenesis of mucopolysaccharidosis IIIC was further proven by expression studies in patient`s fibroblast cell lines.

The article „Mutations in *TMEM76** cause mucopolysaccharidosis IIIC (Sanfilippo C syndrome).“ was published in 2006 in The American Journal of Human Genetics (IF = 12,629).

CONCLUSIONS

This PhD thesis introduces application of novel genomic techniques, especially analysis using DNA array technology and next generation whole exome sequencing. These methods were used in studies of several rare inherited neurological, hepatologic, mitochondrial and lysosomal storage diseases.

The main results of this work include:

1a. Identification of mutations in *DNAJC5* gene as the cause of the adult onset form of neuronal ceroid lipofuscinosis (ANCL), by use of linkage analysis, differential gene expression analyses, copy number variation analysis and whole exome sequencing.

1b. Identification of mutation in *PSEN1* gene and of polymorphism in *CTSD* gene in a family with early-onset Alzheimer disease, which was misclassified and included originally to a set of cases of ANCL, by use of linkage analysis and whole exome sequencing.

2a. Development and optimization of a custom DNA array for copy number variation analysis. This array was used to confirm different molecular basis of two rare conjugated hyperbilirubinemia – Rotor syndrome and Dubin-Johnson syndrome.

2b. Identification of deletions and mutations in *SLCO1B1* and *SLCO1B3* genes, which cause Rotor type of hyperbilirubinemia, by use of copy number variation analysis and homozygosity mapping.

3a. Development and optimization of a custom oligonucleotide DNA array for studies of gene expression in mitochondrial and lysosomal storage diseases (h-MitoArray) and its use for a study of cases with isolated defect of ATP synthase.

3b. Identification of mutations in *TMEM70* gene as a cause of isolated ATP synthase deficiency and neonatal mitochondrial encephalocardiomyopathy by use of linkage analysis, gene expression analysis and homozygosity mapping.

4. Contribution to identification of mutations in *TMEM76 (HGSNAT)* gene as a cause of mucopolysaccharidosis type IIIC by use of gene expression analysis.

Part of the results contributed to acquisition of knowledge about new physiological and patophysiological mechanism. Also, new DNA diagnostic methods were introduced. In summary:

1. Definition of the causal gene for adult neuronal ceroid lipofuscinosis helped to characterize basic pathogenetic mechanism of the disorder. The gene identification enabled a DNA diagnostics in affected families and initiated further stratification of patients with ANCL. Furthermore, cellular and animal models for studies of neurodegenerative mechanisms and for a study of CSPα role are being produced.

2. Studies of Rotor syndrome resulted in description of novel mechanism of bilirubin transport. The study has further clinical consequences for pharmacogenetics in connection with hypersensitivity of patients with OATP1B3 and OATP1B1 mutations. In

addition, identification of mutations enables differential diagnostics of conjugated hyperbillirubinemia.

3. Identification of *TMEM70* mutations contributed to knowledge about biogenesis and regulation of ATP synthase function. It allowed differential diagnostics of isolated ATP synthase defects and offered possibility of prenatal and postnatal DNA diagnostics.

4. Based on the identification of the causal gene for mucopolysaccharidosis IIIC, cellular and animal models for studies of *HGSNAT* gene function and for studies of mechanisms of neurological impairment were prepared. These studies led to proposition of a possible therapeutic approach for patients with MPSIIIC. Moreover, prenatal and postnatal DNA diagnostics in affected families was enabled.

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List of author`s publications, presentations, grants and awards

Publications

1. publications forming the base of the thesis:

Noskova, L., V. Stranecky, H. Hartmannova, A. Pristoupilova, V. Baresova, R. Ivanek, H. Hulkova, H. Jahnova, J. van der Zee, J. Staropoli, K. Sims, J. Tyynela, C. Van Broeckhoven, P. Nijssen, S. Mole, M. Elleder & S. Kmoch (2011) Mutations in *DNAJC5*, Encoding Cysteine-String Protein Alpha, Cause Autosomal-Dominant Adult-Onset Neuronal Ceroid Lipofuscinosis. American Journal of Human Genetics, 89, 241-252. IF 11,68

Ehling, R., Noskova , L., Stranecky V., Hartmannova, H., Pristoupilova, A., Hodanova, K, Venke, T. Kovacs, GG., Strobel, T. Niedermuller, U, Wagner, M., Nachbauer, W., Janecke, A., Budka, H., Boesch, S. & Kmoch, S. (2013) Presenile dementia presenting with cerebellar dysfunction in a multigenerational family harbouring the Presenilin1 p.S170F mutation co-segregating with a Cathepsin D polymorphism. Journal of Neurological Sciences, accepted for publication. IF 2,353

van de Steeg, E., V. Stranecky, H. Hartmannova, L. Noskova, M. Hrebicek, E. Wagenaar, A. van Esch, D. de Waart, R. Elferink, K. Kenworthy, E. Sticova, M. al-Edreesi, A. Knisely, S. Kmoch, M. Jirsa & A. Schinkel (2012) Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver. Journal of Clinical Investigation, 122, 519-528. IF 14,152

Hrebicek, M., T. Jirasek, H. Hartmannova, L. Noskova, V. Stranecky, R. Ivanek, S. Kmoch, D. Cebecauerova, L. Vitek, M. Mikulecky, I. Subhanova, P. Hozak & M. Jirsa (2007) Rotor-type hyperbilirubinaemia has no defect in the canalicular bilirubin export pump. Liver International, 27, 485-491. IF 2,559

Cizkova, A., V. Stranecky, R. Ivanek, H. Hartmannova, L. Noskova, L. Piherova, M. Tesarova, H. Hansikova, T. Honzik, J. Zeman, P. Divina, A. Potocka, J. Paul, W. Sperl, J. Mayr, S. Seneca, J. Houstek & S. Kmoch (2008a) Development of a human mitochondrial oligonucleotide microarray (h-MitoArray) and gene expression analysis of fibroblast cell lines from 13 patients with isolated F(I)F(o) ATP synthase deficiency. Bmc Genomics, 9. IF 3,926

Cizkova, A., V. Stranecky, J. Mayr, M. Tesarova, V. Havlickova, J. Paul, R. Ivanek, A. Kuss, H. Hansikova, V. Kaplanova, M. Vrbacky, H. Hartmannova, L. Noskova, T. Honzik, Z. Drahota, M. Magner, K. Hejzlarova, W. Sperl, J. Zeman, J. Houstek & S. Kmoch (2008b) *TMEM70* mutations cause isolated ATP synthase deficiency and neonatal mitochondrial encephalocardiomyopathy. Nature Genetics, 40, 1288-1290. IF 30,259

Hrebicek, M., L. Mrazova, V. Seyrantepe, S. Durand, N. Roslin, L. Noskova, H. Hartmannova, R. Ivanek, A. Cizkova, H. Poupetova, J. Sikora, J. Urinovska, V. Stranecky, J. Zeman, P. Lepage, D. Roquis, A. Verner, J. Ausseil, C. Beesley, I. Maire, B. Poorthuis, J. van de Kamp, O. van Diggelen, R. Wevers, T. Hudson, T. Fujiwara, J. Majewski, K. Morgan, S. Kmoch & A. Pshezhetsky (2006) Mutations in TMEM76* cause mucopolysaccharidosis IIIC (Sanfilippo C syndrome). American Journal of Human Genetics, 79, 807-819. IF 12.629

2. other publications

Hejzlarova, K., M. Tesarova, A. Vrbacka-Cizkova, M. Vrbacky, H. Hartmannova, V. Kaplanova, L. Noskova, H. Kratochvilova, J. Buzkova, V. Havlickova, J. Zeman, S. Kmoch & J. Houstek (2011) Expression and processing of the *TMEM70* protein. Biochimica Et Biophysica Acta-Bioenergetics, 1807, 144-*149.* IF 5.132

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Poster presentations

A. Čížková, R. Ivánek, L. Piherová, L. Nosková, J. Paul, N. Franssen, S. Kmoch, C. Godinot, J. Keijer and J. Houšťek: Gene expression changes induced by restoration of Von Hippel Lindau tumor supressor deficiency. Poster presentation – practical course Microarray Gene Expression Analysis: Power and Potential of Standardisation, 2005.

A. Čížková, V. Stránecký, R. Ivánek, H. Hartmannová, L. Nosková, L. Piherová, M. Tesařová, H. Hansíková, T. Honzík, J. Zeman, J. Paul, J. Houštěk, S. Kmoch: Human mitochondrial microarray (h-MitoArray) and gene expression analysis in patients with mitochondrial ATP synthase deficiency. Poster presentation - 57th Annual Meeting of the American Society of Human Genetic, 2007.

A. Čížková, V. Stránecký, J.A. Mayr, M.Tesařová, V. Havlíčková, J. Paul, R.Ivánek, A.W. Kuss, H. Hansíková, V.Kaplanová, M.Vrbacký, H.Hartmannová, L.Nosková, T.Honzík, Z.Drahota, M.Magner, K.Hejzlarová, W.Sperl, J.Zeman, J.Houštěk & S.Kmoch: *TMEM70* is a novel factor

of mitochondrial ATPase biogenesis and its mutations cause isolated enzyme deficiency and neonatal encephalo-cardiomyopathy. Poster presentation - 49th Annual Short Course in Medical and Experimental Mammalian Genetics, 2008.

Noskova L., Liskova P., Stranecky V., Hartmannova H., Ivanek R., Jirsova K., Merjava S., Filipec M., Kmoch S.: Posterior polymorphous corneal dystrophy – copy number, gene expression and candidate gene analyses within the PPCD1 candidate region on chromosome 20p 11.2. Poster presentation - FEBS congress 2009.

A. Čížková, V. Stránecký, J.A. Mayr, M.Tesařová, V. Havlíčková, J. Paul, R.Ivánek, A.W. Kuss, H. Hansíková, V.Kaplanová, M.Vrbacký, H.Hartmannová, L.Nosková, T.Honzík, Z.Drahota, M.Magner, K.Hejzlarová, W.Sperl, J.Zeman, J.Houštěk & S.Kmoch: *TMEM70* is a novel factor of mitochondrial ATPase biogenesis and its mutations cause isolated enzyme deficiency and neonatal encephalo-cardiomyopathy. Poster presentation - FEBS course „Mitochondria in Life, Death and Disease“, 2009.

Noskova L., Liskova P., Stranecky V., Hartmannova H., Ivanek R., Jirsova K., Merjava S., Filipec M., Kmoch S.: Posterior polymorphous corneal dystrophy – copy number, gene expression and candidate gene analyses within the PPCD1 candidate region on chromosome 20p 11.2. Poster presentation - 59th Annual Meeting of the American Society of Human Genetic, 2009.

Nosková L., Hartmannová H., Stránecký V., Vepřeková L., Kmoch S.: Identification and characterization of copy number changes: case study of familiar hemoglobinopathy. Poster presentation – 25. pracovní dny – DĚDIČNÉ METABOLICKÉ PORUCHY, 2010.

Nosková L., Hartmannová H., Stránecký V., Vepřeková L., Kmoch S.: Identification and characterization of copy number changes: case study of familiar hemoglobinopathy.Poster presentation – 35th FEBS Congress, Molecules of Life, 2010.

Nosková L., Stránecký V., Hartmannová H., Přistoupilová A., Hůlková H., Elleder M., Jahnová H., Kmoch S.: Využití nových metod analýzy genomu ve studiu molekulární podstaty adultní formy neuronální ceroidní lipofuscinózy (NCL4). Poster presentation – 26. pracovní dny – DĚDIČNÉ METABOLICKÉ PORUCHY, 2011.

Nosková L., Stránecký V., Hartmannová H., Přistoupilová A., Hůlková H., Elleder M., Jahnová H., Kmoch S.: Identification of the genetic and molecular basis of adult neuronal ceroid lipofuscinosis (NCL4) using novel genomic methods.Poster presentation - 36th FEBS Congress, 2011.

Nosková L, Stránecký V, Hartmannová H, Přistoupilová A, Barešová V, Ivánek R, Hůlková H, Jahnová H, van der Zee J, Staropoli JF, Sims KB, Tyynelä J, Van Broeckhoven C, Nijssen PC, Mole SE, Elleder M, Kmoch S.: Mutations in *DNAJC5*, Encoding Cysteine-String Protein Alpha, Cause Autosomal-Dominant Adult-Onset Neuronal Ceroid Lipofuscinosis. Poster presentation - The 12th International Congress of Human Genetics and the 61st Annual Meeting of The American Society of Human Genetics, 2011.

Grants and awards

Grants:

GAUK 56907 Identification and characterization of a gene responsible for posterior polymorphous corneal dystrophy, 2007-2008.

GAUK 299911 Identification of the genetic and molecular basis of adult form of neuronal ceroid lipofuscinosis (NCL4) using novel genomic techniques, 2011 – 2012.

Awards:

Bolzano prize 2011, Mutations in *DNAJC5*, Encoding Cysteine-String Protein Alpha, Cause Autosomal-Dominant Adult-Onset Neuronal Ceroid Lipofuscinosis.

Arnold Beckman prize 2010-2011, Mutations in *DNAJC5*, Encoding Cysteine-String Protein Alpha, Cause Autosomal-Dominant Adult-Onset Neuronal Ceroid Lipofuscinosis.