Clinical relevance of the defects of mitochondrial genome and nuclear genes underlying mitochondrial diseases: from epidemiology through diagnostics to pharmacogenomics

Ph.D. theses

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INTRODUCTION

The mitochondrion

Mitochondria are the small energy producer machinery of the eukarote organisms. The endosymbiotic theory explains the origin of it, and how it has became one of the key double membrane organelle of the eukariote cell. The most important role of the mitochondrion is the synthesis of cellular ATP through oxidative phosphorylation (OXPHOS). Besides these mitochondria have a role in many other cellular processes, like apoptosis, aging, defense against ROS, byosinthesis of steroids, detoxification of ammonia and neurodegeneration.

Genetics of the mitochondria

The human mitochondrial genome is a double-stranded circular DNA (mtDNA) of 16,569 basepairs length, coding 13 proteins, which are the components of the OXPHOS, two ribosomal RNAs (12S rRNA, 16S rRNA) and 22 transfer RNAs (tRNA). The mitochondrial genome differs from the nuclear genome from many viewpoints.

Specific properties of the mtDNA: maternal inheritance, except a few reported cases of paternal inheritance; polyplasmy: in every cell, the circular mtDNA is present in 10-100 copies. The cooccurence of intact and mutated mtDNA in a cell is called heteroplasmy; the treshold effect denotes a critical level of heteroplasmy, at which point the function of the cell will be damaged; mitotic segregation and bottleneck effect: during cell division mitochondria get to the daughter cellsfrom the parent cell randomly. Because of the weak mtDNA repair system, mitochondria are susceptible to mutagen effects, and the mutation rate of the mtDNA is 10 times of the nuclear genome.

Mitochondrial diseases

Mitochondrial diseases are the consequence of the primary defect of the mitochondrial function. Mitochondrial as well as nuclear genome defects may cause mitochondrial diseases.

Symptoms vary on wide phenotypic spectrum, especially when the mtDNA is affected. A characteristic feature of mtDNA diseases is that the same mutation may cause

different phenotypes. On the other hand one clinical symptom may be associated with several mtDNA mutations. Not only the phenotype of distinct patients with the same mutation may vary, but also in the same family significant differences may be present. Mitochondrial DNA defects are responsible for approximately 500 monogenic diseases. The mtDNA associated disorders usually affects several organs simultaneously. The estimated prevalence of mitochondrial diseases is 6.57-9.2/100,000. More than 200 pathogenic mutations and several polymorphisms (SNPs) where identified in the mtDNA, which can cause diverse clinical symptoms, and syndromes. The physical distribution of the mutations on the circular mtDNA is not even, there are mutation hotspots on the molecule.

The most frequent mitochondrial genome associated diseases are the followings: MELAS (Mitochondrial Encephalopathy with Lactacidosis and Stroke-like symptoms), MERRF (Myoclonic Epilepsy with Ragged Red Fibers), NARP (Neuropathy Ataxia Retinitis Pigmentosa), LHON (Leber Hereditary Optic Neuropathy), PEO (Progressive External Opthalmoplegia).

Intergenomic Communication

Besides the mtDNA coded proteins the nuclear genome codes about 1500 proteins of the mitochondrial proteome, which is necessary for the proper functioning of mitochondria. Mutations in these genes cause the intergenomic communication defects between the nuclear and mitochondrial genome, which may manifest as defects of mtDNA quantity: mitochondrial depletion syndrome, a severe infant onset disease; anddefects of mtDNA quality: mtDNA multiplex deletions, which cause most frequently PEO.

One of the key genes in mtDNA biogenesis is the *POLG1* (polymerase gamma), if mutated may cause single and multiplex deletions too in the mtDNA. The diseases associated with *POLG1* mutation are most frequently inherited in autosomal recessive fashion, the most typical diseases are for example Alpers-Huttenlocher syndrome or chronic progressive external opthalmplegia. Some of the mutations of *POLG1* are relevant from pharmacogenomic markers (L304R, A467T, G588D, Q879H, T885S, E1143G, Q1236H), which are correlated to valproate (VPA) associated hepatic failure. The mitochondrial aspartyl-tRNA-synthetase coding gene (DARS2) is a recently

discovered member of the intergenomic communication, of which mutations are responsible for the recessively inherited so called LBSL (Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and Lactate Elevation).

The mtDNA as an anthropological marker

One of the characterstics of the mtDNA is the high proportion of coding sequences, but there is 1000 basepairs length hypervariable region, where no genes are present (D-loop). The SNPs in this region are fixed during evolution, since these mutation lack phenotypic consequences, and therefore play an important role in population genetic studies. The genotype of this hypervariable region defines mitochondrial haplogroups. With the application of molecular biology techniques 8 superhaplogroups and within these 25 haplogroups are differentiated. Present-day Hungarians belong mostly to superhaplogroup R.

OBJECTIVES:

The followings where the main objectives of our research:

1. The study of the occurence of the most frequent mtDNA pointmutations (m.3243 A>G; m.8344 A>G; m.8993 T>C,G; m.3460 G>A, m.11778 G>A és m.14484 T>C) and mtDNA deletions in Hungarian population

2. Investigating the genotype-phenotype correlations of mtDNA mutations and deletions

3. Studying gene-gene interactions: identification of the cooccurence of specific mtDNA and nuclear diseases

4. Analysis of the pharmacogenomic role of mtDNA and POLG1

5. Examine the use of „MitoChip" microarray techniquefor clinical diagnostic purposes

PATIENTS AND METHODS

Patients

We have examined 1568 patients for mtDNA mutations, who were suspected to have mitochondrial disorders, based on clinical symptoms and laboratory findings, from which we have received 543 samples from the University of Pécs, Medical School, Department of Medical Genetics, and 252 samples from the National Institute of Environmental Health Department of Molecular Genetics and Diagnostics. Our asmples were collected during the time of January 1999 and March 2014. We have collected sampkles also from north-central and eastern Hungary. The examined patients can be divided in two groups: the first cohort consisting of 1082 patients (595 female and 487 male, average age: 38.6+/-18.2 years), at which mtDNA hotspot regions (m.3243 A>G , m.8344 A>G , m.8993 T>C, m.8993 T>G known pathogenic mutations) and single and multiplex deletions were examined, and the second cohort, consisting of 486 patients (248 female, 238 male; average age: 34.91+/-17.5 years), at which the three primary LHON mutations (m.3460 G>A, m.11778 G>A, m.14484 T>C) were analyzed. At 70% of the examined patients, more than 5 organ systems were affected by the disorder. Leading symptoms were the followings: myopathy, exercise intolerance, ataxia, PEO and psychiatric disorders. Excluding criteria were advanced age and/or autoimmune diseases. The symptoms of suspected LHON patients are characteristic: optic nerve atrophy, rapidly progressive visual disturbance, macula degeneration and scotomas. The entire examined patient gave their informed consent for the molecular examination.

DNA isolation and Biobank storage

For the genetic diagnostics peripheral blood ethylene-diamine-tetraacectic-acid (EDTA) tubes were collected from1206 patients, and mtDNA was extracted from muscle biopsy specimens at 352 patients. DNA extraction was performed with Qiagen Blood Mini and Tissue kits (Qiagen, Valencia, CA, USA), based on the instructions of the manufacturer. The isolated DNA was temporarily held on -20°C, and for longer banking it was placed in a -80°C refrigerator. The DNA and the data of the patients are a part of the NEPSYBANK project, which is a disease-based biobank.

Myopathologic examinations

Muscle biopsy histology was performed in 352 cases. The ragged red fibers were detected with Gömöri trichrome staining, and ragged blue fibers with modified succinate dehydrogenase (SDH) staining. COX staining was used for detecting „COX negative" fibers, and musclefibers with unusual or uneven staining.

PCR-RFLP examinations

PCR-RFLP methodology was used for the detection of the most frerquent pathogenic mutations in the mtDNA hot spot regions (m. 3243 A>G (MELAS); m. 8344 A>G (MERRF); m. 8993 T>C, G (NARP); m. 3460 A>G; m. 11778 A>G, m.14484 C>T (LHON)). After the PCR, the PCR amplicons were digested with specific restriction endonucleases (New England Biolabs, Ipswich, MA, USA, m. 3243 A>G: HaeIII; m. 8344 A>G: BanII; m. 8993 T>C, G: HpaII, AvaI; m. 3460 A>G: BsaHI; m. 11778 A>G: SfaNI; m.14484 T>C: BccI). Quantity One Softwere (Bio-Rad Corp. Hertfordshire, UK) was used to analyse the size of the bands and heteroplasmy ratios.

Detection of mtDNA deletions

For the detection of single and multiplex deletions long PCR technique was performed, at which synthesis was done at 3 and 8 minutes. Quantity One Softwere (Bio-Rad Corp. Hertfordshire, UK) was used for the determination of the size of the products and heteroplasmy ratios. At the applied methodology, the 8 minutes amplification is used for the detection of larger, and the 3 minutes amplification for the smaller mtDNA deletions.

Bidirectional sequencing of mtDNA

Bidirectional sequencing was performed for whole mtDNA examinations and for validation of microarray findings. The relevant segments of the mtDNA were sequenced with ABI PRISM 3500 Genetic Analyzer sequencer (Applied Biosystem). The detected sequences were aligned on the Cambridge Reference Sequence for human mitochondrial DNA (www.blast.ncbi.nlm.nih.goc /Blast.cgi; NC_012920.1).

Sequencing of the entire mtDNA (MitoChip v2.0)

For the analysis of the whole mtDNAthe one-channel microarray GeneChip® Human Mitochondrial Resequencing Array v2.0 (MitoChip v2.0) (Affymetrix, Santa Clara, CA, USA) was used. The results were analysed with the Sequence Analysis Software 4.1 (GSEQ 4.1), which creates the sequences, and sequenograms from the datas, which were aligned on the human mitochondrial reference genome (www.mitomap.org). The detected mutations were validated with Sanger sequencing.

Haplotyping based on mtDNA

The haplogroups of patients were determined with the use of phylotree.org homepage, based on the polymorhisms of HVSI (a 401 basepairs long hypervariable segment 1) haplogroup specific segment.

Statistics

For the determination of the frequency of most prevalent mtDNA mutations, the number of patients carrying pathogenic mutations (index patients and relatives, who carry the mutation) were divided by the number of all examined patients. The 95% confidence interval was calculated by the standard method.

RESULTS

Epidemiologic Studies

The most frequent mtDNA mutations and deletions localized to mutational hotspots were detected at 377 cases (24.04%) from the examined 1568 Hungarian patients. The mtDNA tRNA localized mutation m.3243 (MELAS) was detected at 16 index patients and further 13 family relatives, frequency: 2.68% (95% CI: 0.0219 – 0.0317); whike the mutation m.8344 A>G (MERRF) was detected at 8 index patient and 5 relatives, frequency: 1.20% (95% CI: 0.0087 – 0.0153). The mutations localized to protein coding genes were detected with the following frequency: m.8993 T>C, G (NARP, MILS) the two mutation was present at 5 patients with a frequency of 0.46% (95% CI: 0.0025 – 0.0067); the three primary LHON mutations m.3460G>A, m.11778 G>A, m.14484 T>C, was detected in 81 patients from the examined 486 patients, which means a frequency of 16.68% (95% CI: 0.1501 – 0.1839).

Single and multiplex deletions were detected in 250 cases from the 1082 patients (23%). At 68 patients we collected blood and muscle biopsy specimen too. From this group of patients, at 6 patients the single deletion was detected from blood as well as from muscle, at 31 cases we did not detect deletion from the blood specimen, although from the muscle biopsy specimen we could identify the deletion (at 16 patients common deletion, and at 15 patients multiplex deletions).

Genotype-phenotype correlations

The most frequently associated phenotype to m.3243 A>G mutation are stroke-like episodes, contrarilythe patients who carried this mutation in our cohort most frequently had neuropsychiatric disorders (depression, psychosis, mental retardation), as well as sensorineural hypoacusis and diabetes mellitus. The most commonly associated symptom to m.8344 A>G is myoclonus epilepsy, although in our cohort only one patient had the classical phenotype, and the most prevalent sypmtoms were ataxia and myopathy.

The variability of clinical symptoms in patients with mtDNA mutations may be the result of different heteroplasmy ratios, which may be different also in the various organs. For example a patient with the mutation m.3243 A>G in a heteroplasmy ratio of

15% may have clinical symptoms, while another patient with the mutation m.8993T>C with a heteroplasmy ratio of 80% may be symptomless.

The mitochondrial common deletions are most frequently correlated to PEO, Kearns-Sayre and Person's syndrome in the literature, but in our cohort it was most frequently associated to myopathy. The mtDNS mutations are usually sporadic, but in one 9 years old patient with Toni-Debré-Falconi syndrome a maternally inherited common deletion was identified. (Heteroplasmy ratio of the index patient: 35%, mother: 25%, grand-aunt on the mothers side: 20%, grandmother on the mathers side: 15%)

Coexisting mtDNA defects

The mtDNA deletions were combined with other mtDNA defects in several cases. Two female patients with m.3243 A>G mutation also carried a large (7.9 kbps) single mtDNA deletion, and a multiplex deletion. At one male patient with the pathogenic m.4298 G>A mutation (tRNA^{Ile}), we have detected a combination with multiplex deletion.

Cooccurence of mtDNA and nuclear gene defects

MtDNA deletions were combined also withother copy number defects. Examination of onehundred patient carrying *PMP22* duplication / deletion (43 *PMP22* deletion and 57 *PMP22* duplication) revelaed mtDNA deletion in 12% of the cases (95% CI: 0.0875-0.1525). In eleven cases a 7.8 kbp single deletion (between 17 and 75% of heteroplasmy ratios), and in one case multiplex deletion was detected with low heteroplasmy ratio. From onehundred Huntington's disease patient with abnormally high CAG repeat who were also evaluated for mitochondrial mutations, 5 female and 2 male (7 cases, 7%, CI: 0.0445-0.0955) carried a single large deletion of 7.9 kbps, with various heteroplasmy ratios between 13% and 75%. We did not detect multiplex deletion in this group.

First case in Hungary of a recently identified mitochondrial disease, determined by a nuclear gene

We were the first in Hungary, identifying a patient with LBSL (leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation) and the underlying

DARS2 mutation in a male patient of age 24 years, whose symptoms started at the age 15 years with gait difficulty. The characteristic appearance of the MRI raised the suspition of the disease, which was later confirmed genetically. A heterozygosus pathogenic splice site mutataion was revealed in the Intron 5-6: c.492+2 T>C (rs142433332; IVS5+2 T>C).The segregation analysis of the family detected this mutation also at the sister, and at the mother of the index patients. The sister has mild symptoms, but the mother is symptomless.

The pharmacogenomic role of mtDNA in metformin side effects

The combined occurence of some of the homoplasmic SNPs of the mtDNA may be associated with elevated risk forcertain disorders. We would like to draw the attention on a case of a 58 years old male, by whom the side effect of metformin may be correlated to variations in the mitochondrial genome. At this patient type 2 diabetes mellitus was diagnosed at the age of 30 years, after which he received metformin, with a 8-10 kgs weight gain in the following 2-3 months. Later insulin therapy was introduced, but because of poor blood glucose levels, the insulin dose was stepwise increased, and after a year he needed 300 IU/day. Creatine kinase levels were slightly elevated, 311 U/L. The patient came to our department with generalised muscle pain, muscle weakness, and the feeling of strain in the skin. Moderate physical activity aggravated muscle pain. During laboratory examinations a lactic acidosis was revealed, the lactate level was 6.6 mmol/l at rest. Two days after the cessation of metformin lactate levels dropped to 4.4 mmol/l, and one month later the lactate was 2.2mmol/l. In parallel physical symptoms remitted markedly, and the insulin dose could be decreased to 97 IU. The molecular genetic examination of this patient revealed common deletion in the mtDNA, in 12% heteroplasmy ratio. Among the identified 32 homoplasmic SNPs, by whole mtDNA resequencing, three (m.1888G>A, m.4216 T>C, m.4917 A>G) is associated with the maternally inherited diabetes and deafness (MIDD).

The pharmacogenomic role of the *POLG1* gene

Detecting a multiplex mtDNA deletion in a patient (65 patients in our cohort) raises the suspicion of intergenomic communication defect, and thus we analyse *POLG1* gene in these patients, since this is the most commonly responsible gene based on the literature.

Until know 7 POLG1 variation is known to be associated to valproate toxicity in the literature (L304R, A467T, G588D, Q879H, T885S, E1143G, Q1236H).From these mutations we have detected 3: one pathogenic mutation (A467T), one modifier variant (E1143G) and one polymorphism (Q1236H), these substitutions were detected in 22 cases from 65 patients (frequency: 33.85%; 95% CI: 0.2798 – 0.3972). The pathogenic mutation with pharmacogenomic role was detected in one patient (1.54%), the nonpathogenic modifier factor was found in 9 cases (13.85%), and the polymorphism was identified at 12 patients (18.46%).

MitoChip v2.0 microarray

Wedesigned a study to compare the microarray with the Sanger-sequencing, regarding time- and cost efficiency. We have examined 15 patients with the microarray, among those 4 patients had known mtDNA mutation, the m.8344 A>G (monozygous male twins) and the m.8933 C>T mutation (mother and her son). At the remaining 11 patients we did not detect pathogenic mutations in the mitochondrial hot spot regions, but clinical symptoms, laboratory results, myopathologic examination and family anamnesis indicated a mitochondrial disease. The mitochip datas were analyzed with the freely downloadable software GSEQ 4.1 (www.affymetrix.com), and they were aligned to the human mtDNA reference genome (www.mitomap.org). The suspected pathogenic mutations were validated with Sanger sequencing, 66% of the detected mutations on mitochip were confirmed with Sanger-sequencing. At every patient several variance were detected, which raised the suspicion of substitutions. During the validation, frequently we could not detect mutation at those positions, thus the methodology is not reliable in detecting heteroplasmic mtDNA mutations in every case. With the help of the detected homoplasmic SNPs we performed the haplotyping of the patients, from 15 patients at one patient the haplogroup (D) was identified, which is characteristic to present day Asian people, while the remaining patients belonged to European (H, U, T, J) haplogroups.

CONCLUSIONS

With the studyof mtDNA and nuclear DNA disorders behind mitochondrial diseases we made the following conclusions:

1. We were the first to perform epidemiologic studies in mitochondrial diseases caused by mtDNA mutations in Hungary, in the setting of a retrospective study. In the examined 1568 patients the frequency of the most prevalent mtDNA point mutations and deletions are: m.3243 A>G 2.68%, m.8344 A>G 1.20%, m.8993 T>C,G 0.46%, m.3460 G>A, m.11778 G>A and m.14484 T>C altogether 16.7%, mtDNA deletions 23.0%, which is similar to others findings in the Caucasian ethnical group. We think that it is of great importance that the genetic diagnostics should be performed not only from blood samples, but from postmitotic tissues as well.

2. With the study of genotype-phenotype correlations of certain mtDNA mutations and deletions, we have found that occasionally unusual phenotypes may be associated to a mutation, and also, between family members symptoms may vary significantly. At certain mutations, like m.8993 T>C relatively high heteroplasmy ratio detected from blood samples may be without symptoms. At other mutations, like m.8344 A>G not only heteroplasmy ratios but also epigenetic effects influence markedly the symptoms, and the course of the disease.

3. We were the first to notice that mtDNA deletions frequently co-occur with other pathogenic mutations in the mtDNA or nuclear genome. We think, that behind this finding may lie insufficient functioning of the DNA replication defect repair mechanisms, and we plan to further investigate this field.

4. We have proved the pharmacogenomic role of mtDNA homoplasmic SNPs in metformin associated side effects.

5. We have supported the importance of testing certain *POLG1* gene SNPs in patients with multiplex deletion, before initiating valproate treatment.

6. We have qualified the MitoChip v2.0 as suitable for detection of mtDNA homoplasmic SNPs, haplotyping, and detection of known mutations, but we do not recommend this methodology for genetic diagnostics of mtDNA diseases.

List of publications

Papers relevant to the dissertation

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