Analysis of intergenomial effects in mitochondrial dysfunction

Doctoral thesis

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INTRODUCTION

Mitochondrion

The mitochondrion is the powerhouse of the cell. The mitochondrion is an organelle that has its own DNA and is inherited only from the mother. During evolution, the mitochondrion became unable to function independently from the nucleus. Mitochondrial proteins required for the mitochondrial functions are mainly encoded in the nuclear DNA.

Mitochondrial diseases can be caused by mutations in mtDNA or nDNA. Genes involved in mtDNA replication and transcription are encoded in nDNA, and the genetic codes of only two rRNA can be found in mtDNA. Though many molecular components required for translation are encoded in mitochondria, the nucleus now holds the key role in this process. While mtDNA mutations show maternal inheritance, nDNA defects have AD, AR or X-linked inheritance patterns. The mtDNA mutation rate is 10 to 20-fold greater than nDNA, thus it is more liable to oxidative damage than nDNA.

Mitochondrial diseases evolve due to a nuclear defect in approx. 80 per cent of patients. In such cases, the disease generally is more severe and the symptoms manifest earlier. Intergenomic communication disorders – examined in my work – are a separate class of mitochondrial diseases that occur when the mtDNA is damaged qualitatively (single or multiple deletions) or quantitatively (depletion). Replication or transcription mechanisms and damage of mitochondrial integrity play a role in its background.

Some evolutionarily conserved mtDNA polymorphisms, particularly deletions have been identified in certain populations. The 9-bp deletion is an East Asian anthropology marker, with the highest incidence in Southeast Asia, Indonesia and the Pacific region. In Europe only three cases have been described.

Evolution of diseases due to mitochondrial dysfunction

The prevalence of mitochondrial diseases are 1 in 5000. Within this heterogeneous group of multisystemic diseases, the skeletal muscle and nervous system are particularly affected. When mitochondrial problems occur, the most energy-intensive tissues are exposed to major damage (brain, retina, muscle, kidney), which are characterized as neuromuscular and metabolic disorders. The apoptosis process changes leading to typical "premature aging".
The multiple deletions are mostly caused by nDNA mutations, which manifest as intergenomic communication disorder. The typical symptoms are myoclonus, ataxia, dementia, neuropathy, ophthalmoplegia, hearing loss, myopathy, hypotonia and atrophy.
Damage to mitochondria plays an important role in neurodegenerative diseases. Energy production decreases, the amount of reactive oxidative free radicals increases, as well as the membrane and the mtDNA is damaging In my study I examined the aKGDH enzyme, which plays a role in the evolution of Alzheimer disease and is an important participant of the Krebs cycle within the mitochondrial matrix.
The most frequently described metabolic abnormality of patients suffering from autism spectrum disorder is mitochondrial dysfunction. An increase in OXPHOS damage and MD biomarkers were detected in ASD-MD. There are many symptoms associated with the ASD phenotype, such as delayed development, loss of various skills and myopathy.

AIMS

The aim of my research was to identify and investigate genes that heavily influence the function of mitochondrium and could be in the background of rare and frequent diseases.

The main objectives:

1. To get a better understanding of the background of mitochondrial dysfunction in neurodegenerative Alzheimer's disease (analysis of genetic alterations in aKGDH)

2. To analyse the genetic variations in neurodevelopmental (autism) disease, and to reveal any possible cause-effect relationship

3. To identify nDNA genes in the background of intergenomial communication disturbancies (mtDNA deletion and depletion)

4. To determine the presence and function of mtDNA 9-bp deletion (East Asian anthropology marker) in the Hungarian population

METHODS

Patients

Alzheimer disease (AD):

Post mortem brain tissues were analysed in 11 Alzheimer patients (6 female, 77±8 ys. and 5 men 69,5±9 ys.) from different regions. Control brain tissue samples were examined (5 female and 4 male, 62±15 ys.) to confirm the pathogenicity of aKGDH mutations.

Mitochondrial disease (MD):

Intergenomial *POLG* gene was sequenced in 131 patients (47 male, 84 female, 40±22 ys.). In selected subcohorts, further genes (*TWINKLE, TK2, RRM2B, ANT1*) were sequenced using Sanger sequencing. An NGS intergenomial panel was tested in 46 patients.
1073 patients with suspected mitochondrial disease (647 female, 44,3±18,5 ys., 426 male, 39,9±19 ys.) and 468 healthy controls were tested (301 female, 38,7±14,4 ys., 167 male 42,7±18,1 ys.) to determine the importance of the 9-bp deletion East-Asian antropological marker.

Autism spectrum disorder (ASD):

ASD children were screened for mitochondral deletion (54 male, 6 female, 10.4±7.3 ys.) Healthy controls were also screened (26 female, 34 male, 28.5±7.4 ys.).

Methods

DNA was isolated from blood, muscle and brain with "QIAamp DNA blood/tissue kit" according to manufacturer's instructions. Long PCR was used to screen for mtDNA deletion. One of the most common mitochondrial SNV (m. 8344 A>G) was tested with PCR-RFLP, digested with BanII endonuclease. Fragments were visualized with ethidium-bromide on 4% agarose gel, evaluated with QuantityOne Software. Muscle specimens were screened for mtDNA depletion using qPCR with Taqman probes (ddCt method).

Sanger sequencing of intergenomial genes (*POLG, TWINKLE, ANT1, TK2* and *RRM2B)* and aKGDH subunits was performed with ABI PRISM 3100 GeneticAnalyzer. Data were analyzed with 3100 Data Collection Software and SequenceScanner (v.1.0), and aligned to human genome reference sequence (National Center for Biotechnology Information, Basic Local Alignment Search Tool).

Next generation sequencing was performed with SureSelect QXT Kit (Agilent Technologies) according to the manufacturer's instructions on MiSeq instrument (Illumina). Analysis was performed with Sure Call software, fastq file alignment with BWA MEM algorithm, and SNP call with SNPPET program. Pathogenic or likely pathogenic mutations from NGS data were validated by Sanger sequencing and segregation analysis was performed in the family. Statistical analysis was used to determine the significance difference between patient and control groups using Chi^2-probe (corrected by Yates).

RESULTS

Alpha-ketoglutarate dehydrogenase alterations and Alzheimer disease

We identified 3 missense mutations in different post mortem brain regions of 11 AD patients. In the *OGDH* gene, a Ser55Leu (c.164 C>T, rs2230445) mutation (Polyphen 0.89, MAF 0,002) was detected only in the temporal region. It was not detected in control samples or in the frontal brain region. In the *DLST* gene, a Pro204Leu (c. 611 C>T, rs142872233) mutation was also found in one control sample, which we classified as a polymorphism. In the *DLD* gene, an Arg263His (c.788 G>A, rs145670503) mutation was detected in three brain regions, but was not found in any of the control samples. Based on various prediction software scores we suspect this to be pathogenic.

Intergenomial communication defects - Sanger sequencing

We detected 267 mtDNA deletions in 1477 suspected mitochondrial disease patients; within different subcohorts we screened pathogenic alterations in intergenomial genes. In 47 patients we observed14 cases which had a decreased number of mitochondrion. ***POLG*** gene analysis: in 131 patients we found 7 pathogenic mutations in 6 patients (Ser204Pro, Gly365Glu, Trp748Ser, Tyr955Cys, Ala1082Thr, Ala467Thr and Cys1197Arg). ***TWINKLE*** gene analysis: in 48 patients, 47 patients were negative. Only one patient harbored 2 mutations (Asn399Ser pathogenic mutation associated with Perrault-syndrome, Arg453Gln unknown mutation). These two alterations could be responsible for the dysfunction of the *TWINKLE* gene in a compound heterozygote form. Using segregation analysis we found an Asn399Ser mutation in the patient's mother and an Arg453Gln mutation in the patient's father. Both parents have no sign of disease. Proband's symptoms: sensorineural hearing loss, ataxia, ovarium dysgenesis, psychiatric symptoms. ***RRM2B*** gene analysis was positive in one family out of 41 cases. A heterozygous c.979 C>T mutation (R327X) was detected with the adPEO phenotype. An adPEO-associated *RRM2B* mutation has not been described in the literature.

Intergenomial communication defects – Next generation sequencing

Next-generation sequencing was performed on 36 patients (27 patients with mtDNA deletion and 9 patients with mtDNA depletion). In 2/3 of the cases, we found likely pathogenic alterations. In 3 cases, we described the known pathogenic Arg178Trp mutation of the exonuclease MGME1(mitochondrial genome maintenance exonuclease), which is responsible for the integrity of the mitochondrial genome. Its pathogenicity was confirmed by segregation analysis. We also found previously published pathogenic mutations in the SUCLG1 gene (Gly79Asp), which catalyses the Krebs cycle, and in MTO1 gene, (Thr308Ala), which plays a role in protein translation. Furthermore, we also identified a mutation in the MRPL3 gene (Ser75Asn) coding the mitochondrial ribosomal protein. We determined the importance of many previously undescribed mutations. We did not find pathogenic depletions in 5 of 9 children. We found a homozygote MGME1 Arg178Trp pathogenic mutation in the remaining four children.

Mitochondrial dysfunction in autism

In 60 ASD patients, we identified a mitochondrial deletion in 10 cases. In 60 healthy controls, mtDNA deletion was found in 2 cases. The results showed significant difference between autistic and control cohorts (Yates' chi^2 4.5; p=0.03). MtDNA alteration is proven in 16.6% of the investigated patients. In this cohort, we detected only one case with the syndromic form of ASD. I focused on MD-ASD patients (autism with mitochondrial deletion) in my work. In *Patient3* we found a heterozygous pathogenic mutation in the *CHD7* gene. Besides this mutation, a heterozygous *TSC2* mutation with uncertain significance was also detected. The patient's phenotype and the family segregation pattern indicated that the *CHD7* gene mutation resulted in CHARGE syndrome.

In the 10 MD-ASD cases, 4 patients have known pathogenic mutation(s) in heterozygous forms, either in ASD associated genes or in genes responsible for mtDNA maintenance. In 6 cases, mutations with uncertain significance have been found following in silico analysis of genes responsible for intergenomial communication. Affected intergenomial genes include *MGME1, POLG* and *SUCLG1*. In most of the patients, we didn't find any alterations in the intergenomial panel. However, we did find them in ASD-associated genes, with direct or indirect correlation to mitochondrial dysfunction.

Importance of the East-Asian anthropological marker

We identifed the 9-bp deletion in 14 cases among 1073 patients with mitochondrial dysfunction. Among 468 healthy control individuals, only one person harbored the 9-bp mtDNA deletion. The mutation frequency of the 9-bp deletion in our Hungarian mitochondrial patient cohort (in central Europe, Caucasian population) is 1.3% (14 cases out of 1073 patients), and in the control group is 0.2% (one case out of 468 controls). In 203 of the 1073 total patients, the mtDNA disease was genetically proven, it means that all of them had a pathogenic mutation in the mtDNA. In this 203-patient cohort, we found ten cases harboring the 9-bp deletion. If we compare statistically the co-prevalence of the 9-bp deletion with pathogenic mtDNA mutations (ten cases out of 203 patients) to normal controls (one case out of 468 healthy individuals) the difference is statistically significant ($p=0.00004$; Yates' chi-square 16.69).

In one family (three patients), in addition to these alterations we found a new pathogenic heteroplasmic m.8332 A>G mutation on the $tRNA^{Lys}$ anticodon stem. In five cases the 7.9 kb common deletion was present, in one case multiple deletions were detected. In one patient we found a frame shift mutation in the D-loop. In four mitochondrial cases harboring the 9-bp deletion we did not find any pathogenic mutations in the mitochondrial genome.

CONCLUSIONS

We were looking for aKGDH mutations in post mortem brain tissues diagnosed with Alzheimer's disease. We detected a 1-1 missense mutation in each of three subunits. We hypothesize that two of them are pathogenic (OGDH and DLD subunit) and one of them (DLST subunit) is a polymorphism. Alterations of aKGDH could be risk factors for AD.

We identified previously unpublished missense mutations with uncertain significance in different intergenomial communication genes by next generation sequencing. We described predictions and determined the importance of these mutations based on segregation and in silico analysis. We found one family with compound heterozygous *TWINKLE* mutations associated with Perrault-syndrome.

We conclude that intergenomial genes (*POLG, RRM2B, MGME1*) could be inherited in autosomal dominant (mild phenotype, mtDNA deletion) or recessive form (more severe symptoms, mtDNA depletion). We identified an *MGME1* Arg178Trp alteration in several cases (result of segregation analysis: de novo mutation).

In this study we provide for the first time a comprehensive genetic analysis of ASD patients after identifying the most common syndromic forms of ASD. The coexistence of the most frequent mtDNA alterations, intergenomial communication disturbances (51 genes) and genes previously associated with ASD have been simultaneously analyzed. Among patients with ASD, the presence of mtDNA alteration is more common than in control individuals. In our work we highlight the mitochondrial associations of ASD genes. We detected alterations in genes, in direct or indirect context to mitochondrion in all MD-ASD cases. The affected genes function in cell regulation and signal transduction. The mtDNA deletion is usually not a single genetic alteration in ASD, it coexists both in syndromic and non-syndromic ASD forms either with other ASD associated genetic risk factors and/or alterations in genes responsible for intergenomial communication. These findings indicate a very complex pathophysiology of ASD in which mitochondrial dysfunction is an important key player.

Among European populations we also determined the incidence of a 9-bp deletion East-Asian anthropology marker in Hungary. Our findings confirm the hypothesis that this deletion causes mitochondrial DNA instability and we identify it as an MD risk factor.

PUBLICATIONS

Related publications:

Gal A, **Pentelenyi K**, Remenyi V, Pal Z, Csanyi B, Tomory G, Rasko I, Molnar MJ. (2010) Novel heteroplasmic mutation in the anticodon stem of mitochondrial tRNA(Lys) associated with dystonia and stroke-like episodes. Acta Neurol Scand, 122(4):252-6. IF: 2.153

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Reményi V, **Pentelényi K**, Valikovics A, Mede K, Szegedi N, Szilágyi G, Óváry Cs, Nagy Z, Gál A, Molnár MJ. (2010) Thrombocytamembrán-glikoprotein receptor polimorfizmusainak vizsgálata a fiatalkori ischaemiás stroke szindróma hátterében. Vascularis Neurologia folyóirat, 2(1):27-32.