DOINA ȚURCAN*, DANIELA BLĂNIȚĂ, NATALIA UȘURELU, VICTORIA SACARĂ*

# METHODOLOGICAL APPROACHES IN THE MOLECULAR GENETIC ANALYSIS OF MITOCHONDRIAL DNA IN PATIENTS WITH COMMON CLINICAL FEATURES OF MITOCHONDRIAL DISEASE

*Institute of Mother and Child, Chisinau, Republic of Moldova*

**REZUMAT**

## ABORDĂRI METODOLOGICE ÎN ANALIZA MOLECULAR-GENETICĂ A ADN-ULUI MITOCONDRIAL LA PACIENȚII CU SIMPTOMATOLOGIA CARACTERISTICĂ DEREGLĂRILOR MITOCONDRIALE

**Introducere.** Bolile mitocondriale sunt una dintre cele mai frecvente erori înnăscute ale metabolismului, cu o prevalență estimată de aproximativ 1: 5.000. Disfuncția mitocondrială poate apărea din cauza unor defecte ale genelor din ADN-ul mitocondrial și nuclear care codifică proteine mitocondriale structurale sau cele implicate în funcția mitocondrială.

**Scopul:** Detectarea și cuantificarea heteroplasmiei ADN-ului mitocondrial la pacienții cu simptomatologia caracteristică dereglărilor mitocondriale.

**Materiale și metode.** Studiul a implicat un grup de cercetare format din 20 de pacienți cu simptomatologia caracteristică dereglărilor mitocondriale și 10 persoane sănătoase ca grup de control. Tehnica ARMS PCR a fost realizată pentru toate probele din grupul de studiu și din grupul control. Ulterior, s-a aplicat tehnica ARMS-qPCR doar în cazurile în care mutația a fost detectată prin tehnica ARMS PCR.

**Rezultate.** Tehnica ARMS-PCR, efectuată la ambele grupuri de cercetare, a determinat prezența mutației 3243A>G la toate persoanele din grupul de studiu și cel de control și mutația 3460G>A la 4 pacienți din grupul de studiu. Datele preliminare cu privire la analiza heteroplasmiei ADN-ului mitocondrial au relevat un nivel înalt de heteroplasmie (55-78%) la 5 pacienți cu mutația 3243A>G și la 4 pacienți cu mutația 3460 G>A, fiind în concordanță cu semnele clinice.

**Concluzii.** Metoda ARMS-qPCR este o tehnică simplă, eficientă din punctul de vedere al timpului și costului, comparativ cu alte metode.

**Cuvinte-cheie:** ADN mitocondrial, mutații punctiforme, ARMS-qPCR, heteroplasmie.

**РЕЗЮМЕ**

## МЕТОДОЛОГИЧЕСКИЕ ПОДХОДЫ В МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКОМ АНАЛИЗЕ МИТОХОНДРИАЛЬНОЙ ДНК У ПАЦИЕНТОВ С КЛИНИЧЕСКИМИ ПРОЯВЛЕНИЯМИ МИТОХОНДРИАЛЬНОЙ БОЛЕЗНИ

**Введение:** Митохондриальные заболевания являются одним из наиболее распространенных врожденных нарушений обмена веществ, с распространенностью приблизительно 1: 5000. Митохондриальная дисфункция может возникать из-за дефектов генов в митохондриальной и ядерной ДНК, которые кодируют структурные митохондриальные белки или белки, участвующие в митохондриальной функции. **Цель:** выявление и количественная оценка гетероплазмической мутантной митохондриальной ДНК у пациентов с клиническими проявлениями митохондриальной болезни.

**Материалы и методы:** В исследовании участвовала группа из 20 пациентов с клиническими проявлениями митохондриальной болезни и 10 здоровых персон в качестве контрольной группы. Метод ARMS-PCR был выполнен для всех образцов из исследовательской группы и контрольной группы. Далее, был проведён анализ ARMS-qPCR только в тех случаях, когда мутация была идентифицирована методом ARMS-PCR.

**Результаты:** Метод ARMS-PCR был выполнен у субъектов обеих исследовательских групп и было определено наличие мутации 3243 A> G у всех субъектов в группе исследования и контроля, и мутации 3460 G> A у 4 пациентов исследовательской группы. Предварительный анализ уровня гетероплазмии выявил высокую степень гетероплазмии (55-78%) у 5 пациентов с мутацией 3243 A> G и у 4 пациентов с мутацией 3460 G> A в соответствии с клиническими проявлениями.

**Заключение:** Метод ARMS-qPCR удобен в использовании, экономичен и эффективен по сравнению с другими методами для определения гетероплазмии.

**Ключевые слова:** митохондриальная ДНК, точечные мутации, ARMS-qPCR, гетероплазмия.

**Introduction.**

The mitochondrion is a highly specialized organelle, present in almost all eukaryotic cells and principally charged with the production of cellular energy through oxidative phosphorylation (OXPHOS). In addition to energy production, mitochondria are also key components in calcium signalling, regulation of cellular metabolism, haem synthesis, steroid synthesis and, perhaps most importantly, programmed cell death (apoptosis) [1]. Mitochondrial DNA is a maternally inherited, circular double-stranded molecule of 16 569 base pairs, encoding 37 genes (13 proteins of the OXPHOS system, 2 rRNAs, and 22 tRNAs) [2].

Mitochondrial diseases are one of the most common inborn errors of metabolism, with a conservative estimated prevalence of approximately 1:5,000 [3]. Mitochondrial function is under dual genetic control – the 16.6-kb mitochondrial genome and the nuclear genome, which encodes the remaining ~1300 proteins of the mitoproteome. Mitochondrial dysfunction can arise because of defects in either mitochondrial DNA or nuclear mitochondrial genes, and can present in childhood or adulthood in association with vast clinical heterogeneity, with symptoms affecting a single organ or tissue, or multisystem involvement [2]. Clinical symptoms can arise in childhood or later in life, and can affect one organ in isolation or be multisystemic [4]; the minimum disease prevalence in adults is ~12.5 per 100 000, and ~4.7 per 100 000 in children [3]. Nuclear DNA defects tend to be autosomal recessive and are transmitted in a Mendelian fashion where half of the genetic material of a fertilized egg derives from each parent. Since the egg has multiple mitochondria while sperm has virtually none, mutations of mitochondrial genes in mtDNA are transmitted through the maternal line, or they may be sporadic. The number of mitochondria within cells varies depending on the energy needs of the cells. Cells which have more ATP requirements will have more mitochondria. As an example, muscle cells and heart muscle cells need more energy than the cells of the liver and have more mitochondria to generate more ATP. The heart muscle cells have about 5,000 mitochondria and each liver cell contains 1,000–2,000 mitochondria [5].

Point mutations and large-scale mtDNA deletions represent the two most common causes of primary mtDNA disease, the former usually being maternally inherited, and the latter typically arising de novo during embryonic development [2]. The multicopy nature of mtDNA gives rise to heteroplasmy, a unique aspect of mtDNA-associated genetics that occurs when there is coexistence of a mix of mutant and wild-type mtDNA molecules (heteroplasmy). In contrast, homoplasmy occurs when all of the mtDNA molecules have the same genotype. Heteroplasmic mutations often have a variable threshold, i.e. a level to which the cell can tolerate defective mtDNA molecules [6]. When the mutation load exceeds this threshold, metabolic dysfunction and associated clinical symptoms occur. The degree of mtDNA mutant heteroplasmy can vary significantly across different tissues of the same individual, and the percentage of a mutation is an important contributor to the clinical phenotype [7]. Due to heteroplasmy, mitochondrial disease takes many forms and even family members may present differently with the disease. Determination of the heteroplasmic status of any mtDNA mutation is clinically important to providing a family with informative genetic counseling regarding recurrence risk. Therefore, accurate measurement of heteroplasmy is an essential component of the molecular diagnostic scheme for mtDNA-related disorders. Methods widely used to detect heteroplasmic mutations include Restriction Fragment Length Polymorphism (RFLP), direct sequencing, TaqMan allelic discrimination analysis, Amplification Refractory Mutation System Quantitative Polymerase Chain Reaction (ARMS-qPCR), and PCR/allele-specific oligonucleotide (ASO) dot blot hybridization [8]. ARMS-qPCR is a cost-effective method that measures the relative amount of wild-type and mutant mtDNA in a single step.

The most common mitochondrial diseases are MELAS syndrome (Mitochondrial Encephalopathy, Lactic acidosis, Stroke-like episodes), LHON syndrome (Leber's Hereditary Optic Neuropathy), Leigh syndrome, NARP syndrome (Neuropathy, Ataxia, Retinitis Pigmentosa). The presence of mitochondrial diseases presents a social problem, leading to substantial direct and indirect costs of medical care for the patient and for society as a whole. Genetic testing for mitochondrial diseases is important because it allows early access to standard care, avoiding invasive investigations and significant impact on the evolution of the disease, but also genetic counseling of the family.

**The aim:** Detection and quantification of heteroplasmic mutant mitochondrial DNA in patients with common clinical features of mitochondrial disease.

**Materials and Methods.** Patients were referred to the Human Molecular Genetics Laboratory at the Institute of Mother and Child, for genetic counseling. The most frequent mutations involved in occurrence of mitochondrial disease (MT-TL1–3243A>G, MT-ND1–13513G>A, MT-ND1–3460G>A, MT-ND4–11778G>A, MT-ND6–14484T>C, MT-ATP6–8993T>C, MT-ATP6–8993T>G) were verified by ARMS-PCR technique in a research group of 20 patients presenting common clinical features of mitochondrial disease and 10 healthy persons as control group. Total DNA was isolated from peripheral blood lymphocytes by a salting-out method. The ARMS PCR technique was performed for all samples from the study group and from the control group. ARMS PCR primers are able to discriminate a single nucleotide change on the template DNA due to the absences of 3′ exonucleolytic proofreading activity associated with Taq DNA polymerase. Under appropriate conditions, oligonucleotides containing a mismatched 3′ -residue are unable to function as primers. Introducing a mismatched nucleotide immediately 5′ to the mutation site will further increase target DNA amplification specificity. ARMS primers for PCR analysis of seven common mtDNA point mutations used in the study are listed in Table 1 [9][10][11][12].

Subsequently, the real time ARMS qPCR assay was performed in duplicates for each wild-type and mutant sequence, only in cases where the mutation was identified by ARMS PCR technique. The ARMS qPCR was run in Applied Biosystems™ 7500 Real-Time PCR System using 7500 Software (Version 3.2). The percentage of mutation heteroplasmy was calculated by $\Delta C_T$ ($C_T^{wild\text{-}type} - C_T^{mutant}$). Mutation % = $1/[1+(1/2)^{\Delta C_T}]$ x 100%. The threshold cycle ($C_T$) is the cycle at which a significant increase in the reaction product is first detected. The higher the initial amount of DNA, the sooner accumulated product is detected in the PCR process and the lower the $C_T$ value.

**Table 1. Primer sequences for ARMS PCR**

| Name | Forward/ Reverse | Sequence | Size (pb) |
|---|---|---|---|
| *MT-TL1*–3243A>G | Forward wild-type | AGGGTTTGTTAAGATGGCtcA | 97 |
| | Forward mutant | AGGGTTTGTTAAGATGGCtcG | |
| | Reverse | TGGCCATGGGTATGTTGTTA | |
| *MT-ND1* –13513G>A | Forward wild-type | CTCACAGGTTTCTACTCCAAtG | 145 |
| | Forward mutant | CTCACAGGTTTCTACTCCAAtA | |
| | Reverse | TTCTTCTCACCCTAACAGGTC | |
| *MT-ND1* –3460G>A | Forward wild-type | TACTACAACCCTTCGCTGcCG | 120 |
| | Forward mutant | TACTACAACCCTTCGCTGcCA | |
| | Reverse | GTAGAAGAGCGATGGTGAGAGCTAAG | |
| *MT-ND4* –11778G>A | Forward wild-type | ACGAACGCACTCACAGTgG | 143 |
| | Forward mutant | ACGAACGCACTCACAGTgA | |
| | Reverse | CACAGAGAGTTCTCCCAGTAGGTTAA | |
| *MT-ND6* –14484T>C | Forward wild-type | GTAGTATATCCAAAGACAACgAT | 161 |
| | Forward mutant | GTAGTATATCCAAAGACAACgAC | |
| | Reverse | GGGTTTTCTTCTAAGCCTTCTCC | |
| *MT-ATP6* – 8993T > C | Forward wild-type | TACTCATTCAACCAATAGCCaT | 75 |
| | Forward mutant | TACTCATTCAACCAATAGCCaC | |
| | Reverse | TTAGGTGCATGAGTAGGTGGC | |
| *MT-ATP6* – 8993T > G | Forward wild-type | TACTCATTCAACCAATAGCCaT | 75 |
| | Forward mutant | TACTCATTCAACCAATAGCCaG | |
| | Reverse | TTAGGTGCATGAGTAGGTGGC | |

**Results.**

The ARMS-PCR technique was performed in patients presenting common clinical features of mitochondrial disease and in healthy persons in the control group. It was determined the presence of the 3243 A>G mutation in all persons in the research and control group and the 3460 G>A mutation in 4 patients of the research group.

Preliminary analysis of heteroplasmy level revealed a high degree of heteroplasmy in 5 patients with 3243 A>G mutation, indicating the presence of MELAS syndrome, a multisystem disorder characterized by stroke-like episodes, encephalopathy with seizures and/or dementia, lactic acidosis, muscle weakness and exercise intolerance, recurrent vomiting, hearing impairment, peripheral neuropathy, and learning disability (Fig. 1-3).

MT-ND1 – 3460 G>A mutation was established in 4 patients of the study group, which confirms the presence of LHON syndrome, a disorder with bilateral loss of central vision predominantly in young males (Fig. 4-6).

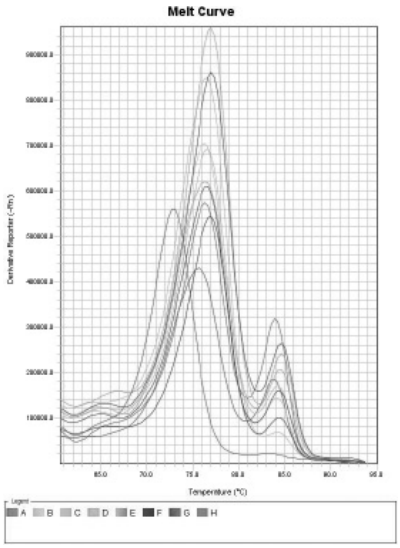


**Fig. 1. Melting curve of internal control - albumin**

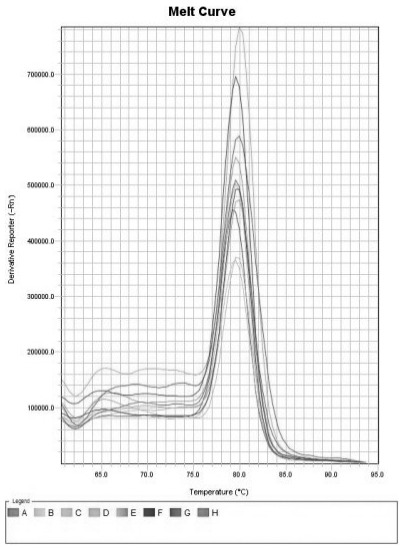


**Fig. 2. Melting curve of mutant primer, 3243 A>G mutation**

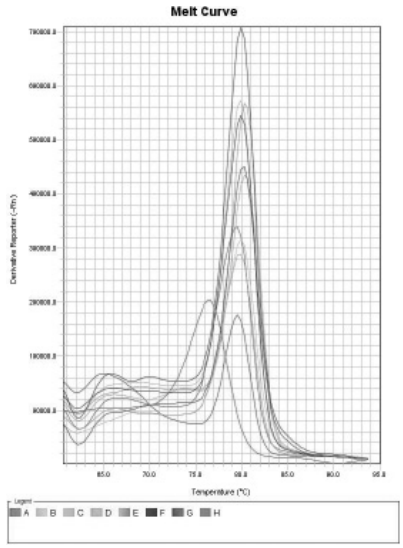


**Fig. 3. Melting curve of wild-type primer, 3243 A>G mutation**

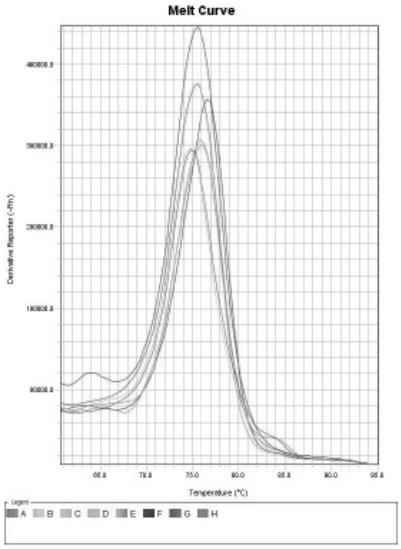


**Fig. 4. Melting curve of internal control - albumin**

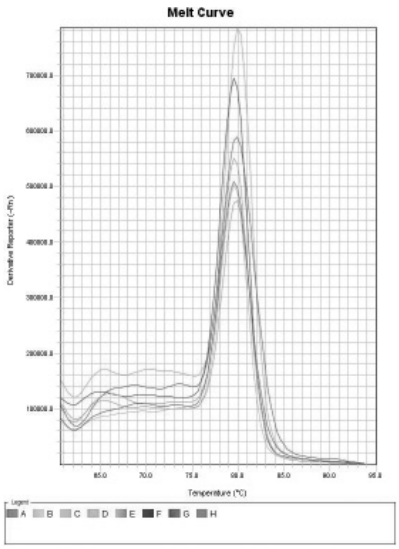


**Fig. 5. Melting curve of mutant primer, 3460 G>A mutation**

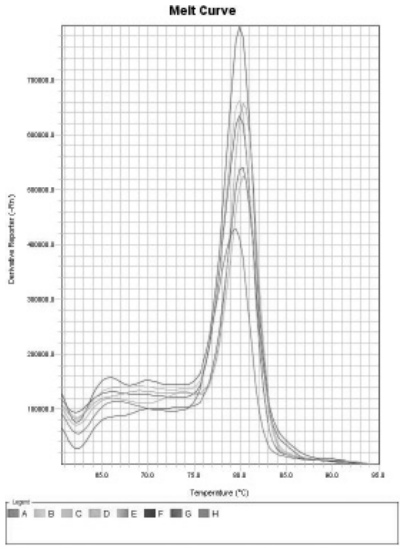


**Fig. 6. Melting curve of wild-type primer, 3460 G>A mutation**

**Conclusions.** The real-time ARMS-qPCR method provides a simple, easy-to-read output that is cost- and time-effective, thus providing an alternative method to individual endpoint PCR-restriction fragment length polymorphism (RFLP) or PCR followed by Sanger sequencing. The ARMS-PCR technique was performed in subjects of both research groups, determining the presence of the 3243 A>G mutation in all persons in the research and control group and the 3460 G>A mutation in 4 patients of the research group. Preliminary analysis of heteroplasmy level revealed a high degree of heteroplasmy (55-78%) in 5 patients with 3243 A>G mutation and 4 patients with 3460 G>A mutation, being in accordance with clinical manifestations. The control group had a low-level of heteroplasmy, which implies the absence of clinical features of mitochondrial diseases.

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