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DNA polymorphisms detected in *MT-ATP6* and *MT-ATP8* genes in the residents of Sarajevo Canton

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Abstract

Human mitochondrial genes *MT-ATP6* and *MT-ATP8* encode the subunits 6 and 8, respectively, of ATP synthase, a vital protein Complex V intricately involved in oxidative phosphorylation and ATP metabolism. This enzyme produces ATP from ADP in the mitochondrial matrix utilizing energy provided by the proton electrochemical gradient. Pathogenic mutations within these genes have been linked to various syndromes such as NARP syndrome, Leigh syndrome, mitochondrial myopathy with reversible cytochrome C oxidase deficiency, and progressive spastic paraparesis, among others. In our investigation, we sequenced 24 complete human mitochondrial genomes of healthy adult individuals from Bosnia and Herzegovina, each representing unique maternal lineage. Employing the Illumina MiSeq NGS platform and the Nextera XT DNA library preparation protocol, we obtained raw NGS reads. Subsequent analysis utilizing SAMtools enabled the identification of genetic variants within the *MT-ATP6* and *MT-ATP8* genes. We identified a total of 11 SNPs, including three in *MT-ATP8* and eight in *MT-ATP6*, with none of them being associated with any mitochondrial diseases or conditions. Our results align well with previously reported genome variation data for European populations and set the groundwork for future mtDNA analysis for clinical purposes in Bosnia and Herzegovina.

Keywords

mtDNA, MT-ATP6, MT-ATP8, NGS, Bosnia and Herzegovina

Introduction

The majority of ATP in eukaryotic cells is generated within the mitochondrion, utilizing a protein complex known as the electron transport chain (ETC). The intricate system, historically termed the mitochondrial oxidative phosphorylation (OXPHOS) system, consists of the four key complexes (I-IV) of the ETC (Houštěk et al., 2006). ATP serves as the primary energy source for most life forms and is a precursor in the synthesis of DNA molecules, signaling molecule and secondary messenger, and a carrier molecule enabling a range of metabolic reactions to occur (Jonckheere et al., 2012).

Within the mitochondrial genome, genes *MT-ATP6* and *MT-ATP8* encode crucial components of the ATP synthase complex. Being located next to each other in the human mtDNA, the final 46 nucleotides of the *MT-ATP8* gene sequence overlap with the beginning of the coding region of *MT-ATP6*. This unique feature of these two genes is quite unusual since the mutations within these overlapping regions have the potential to induce changes in both protein subunits simultaneously (Fragaki et al., 2019).

Mutations in *MT-ATP8* are relatively rare, occurring in only a small percentage of cardiomyopathy patients. Previous research has shed light on the impact of mutations in *MT-ATP8* and other genes on the biochemical pathway of oxidative phosphorylation (Fragaki et al., 2019). These mutations have been predominantly linked to a range of neurodegenerative and cardiovascular disorders with severe clinical presentation; among those conditions, the most commonly investigated ones are Leigh syndrome, Leber's hereditary optic neuropathy (LHON), NARP syndrome (neuropathy, ataxia, retinitis pigmentosa), and mitochondrial encephalomyopathy with stroke-like episodes (MELAS) (Xu et al., 2015). Frameshift mutations in *MT-ATP6* have been associated with onset of ataxia, microcephaly, developmental delay, and intellectual disability. Additionally, certain genetic mutations, such as those in *MT-ATP6* and *MT-ATP8*, are implicated in infantile hypertrophic cardiomyopathy (CMHI) (Jonckheere et al., 2009; Wong, 2007).

Material and methods

Buccal swab samples for DNA isolation were obtained from 24 adult, evenly distributed by gender, healthy, unrelated individuals who provided thorough informed consent prior to participating in the study. All individuals inhabit the territory of Sarajevo Canton. Buccal swab samples were collected in duplicate from each individual using Citotest® - Citoswab® cotton swabs (Wellkang Ltd, Derry, Northern Ireland). The study was approved by the Ethics Committee of the Department of Genetics and Bioengineering, International Burch University, on November 30th 2023, document number: 04-179/23.

Genomic DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The concentration of the extracted DNA samples was quantified using the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) with Qubit® dsDNA Quantitation High Sensitivity kits (Invitrogen, Carlsbad, CA).

The Nextera XT DNA Library Preparation method was used for the next-generation sequencing (NGS) of mtDNA. In our case, the entire mitochondrial genome was amplified in two long-read PCR reactions, each fragment exceeding 8 kb in length.

Library preparation involved several steps, the initial amplification step utilized LA Taq DNA polymerase—for long-range PCR (Takara Bio, Kyoto, Japan). Human mtDNA specific primers used for the first PCR and designed for this study are: MT1F 5'-TCTTTGCAGGCACACTAC-3', MT1R 5'-GGGGGAGGTTATATGGGTTT-3', MT2F 5'-ATGATACGCCCGAGCAGA-3', and MT2R 5'-CAACCGCATCCATAATCCTT-3'.

Quantification of the amplified fragments was conducted using the Agilent 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA), which were approximately 9.8 kb and 8.5 kb in length. The tagmentation step followed the manufacturer's instructions, and for index PCR, IDT® for Illumina® DNA/RNA UD Indexes were used (Illumina, USA). Subsequently, libraries were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). Normalization of the prepared libraries was carried out using the Agilent High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer Instrument (Agilent Technologies, USA). Normalized libraries were pooled, denatured and diluted according to the manufacturer's instructions outlined in the MiSeq System Denature and Dilute Libraries Guide, document #15039740 v10 (Illumina, USA).

Paired-end sequencing was performed on an Illumina® MiSeq instrument using the MiSeq® Nano Reagent Kit v2, 300 cycles (2 x 151 bp). The Local Run Manager Generate FASTQ Analysis Module on the Illumina MiSeq instrument was employed to generate FASTQ files. To produce an interleaved FASTQ file from two separate files containing the forward and reverse reads of paired-end reads, the SeqFu tool was utilized (Telatin et al., 2021). Bowtie2 software (Langmead et al., 2018) mapped the short reads against the revised Cambridge Reference Sequence (rCRS) for human mitochondrion (Andrews et al., 1999), followed by the use of SAMtools to sort the SAM files generated after the mapping pipeline and perform sequence pileup (Danecek et al., 2021). BCFtools was then used to generate base calls and VCF files (Danecek et al., 2021). Quality control of the reads was conducted using FastQC software, and MultiQC was utilized to generate a unified report of the completed sequencing data (Ewels et al., 2016).

Results and Discussion

In 24 human samples, we observed 11 different nucleotide variations in *MT-ATP8* and *MT-ATP6* genes, all of them being single nucleotide polymorphisms (SNPs), with no insertions or deletions (indels) presented in Table 1.

MT-ATP8 presented with three different SNPs in our study, whereby all three were transitions. While m.8410C>T is classified as likely benign in ClinVar (Landrum et al., 2014) and not associated with any phenotype, the remaining two variants, namely m.8448T>C and m.8463A>G were classified as benign in both cases. Certain mutations in *MT-ATP8* gene are associated with Leigh syndrome, also known as subacute necrotizing encephalomyelopathy, a neurological condition which is present worldwide and usually characterized by an early onset in infancy or childhood. Leigh syndrome is described as a complex, heterogenous group of symptoms, previously associated with 16 mitochondrial and around 100 nuclear genes, with *MT-ATP8* being one of them (Rahman, 2023). Considering that our participants are healthy individuals with no reported family history of neurodegenerative diseases, it is reasonable that detected variants truly are benign in this regard.

Table 1. Detected nucleotide variants in MT-ATP8 and MT-ATP6 genes in Bosnian-Herzegovinian population (using information from ClinVar (Landrum et al., 2014) and dnSNP (Sherry et al., 2001) databases).

SNP	Gene	Protein change	Significance (ClinVar)	Number of samples with detected change	Frequency of appearance in this study (%)	Alternative ALFA Allele frequency, Europe
m.8410C>T	*MT-ATP8*	p.Pro15=	Likely benign	1	4.17	0.001
m.8448T>C	*MT-ATP8*	p.Met28Thr	Benign	2	8.33	0.011
m.8463A>G	*MT-ATP8*	p.Tyr33Cys	Benign	1	4.17	0.0002
m.8697G>A	*MT-ATP6*	p.Met57=	Uncertain significance	4	16.67	0.101
m.8706A>G	*MT-ATP6*	p.Met60=	Not reported in ClinVar	1	4.17	Not observed
m.8818C>T	*MT-ATP6*	p.Leu98=	Likely benign	1	4.17	0.006
m.8860A>G	*MT-ATP6*	p.Thr112Pro	Benign	24	100.00	0.989
m.8994G>A	*MT-ATP6*	p.Leu156=	Not reported in ClinVar	2	8.33	0.022
m.9055G>A	*MT-ATP6*	p.Ala177Thr	Benign	5	20.83	0.168
m.9117T>C	*MT-ATP6*	p.Ile197=	Not reported in ClinVar	1	4.17	0.003
m.9151A>G	*MT-ATP6*	p.Ile209Val	Benign	2	8.33	0.007

MT-ATP6 is more polymorphic and in our study population with eight different identified SNPs. However, three of those SNPs were not previously reported in ClinVar (Landrum et al., 2014) as clinically relevant and were therefore not further analyzed in this study. Furthermore, variant m.8706A>G, which we observed in one sample, was also not reported in the ALFA Allele project (Sherry et al., 2001) for the European populations (n = 6060), indicating the need for further research.

Out of the remaining five SNPs, three are benign, one is likely benign and one is of uncertain significance, based on previous ClinVar reports (Landrum et al., 2014). Absence of relevant phenotypes in our participants further confirms such clinical significance of detected variants. Probably the most interesting variant for further research is m.8697G>A (rs879233543), which is listed as associated with Leigh syndrome with unknown significance (Richards et al., 2015), but with no previous publications dealing with its associated clinical conditions. Benign variant m.8860A>G (rs2001031) was previously investigated for its association with male subfertility, however, no statistically significant association were found for this, or any other *MT-ATP6* variant between the study (n = 67) and control (n = 44) groups (Saleh Jaweesh et al., 2022). *MT-ATP6* was previously associated with different conditions, such as Leigh syndrome, NARP syndrome (Baracca et al., 2000; Holt et al., 1990; Kerrison et al., 2000; Sgarbi et al., 2009), and Leber optic atrophy (Lamminen et al., 1995). However, none of the mutations detected in these studies were present in our healthy participants.

The studies of mtDNA protein-coding genes are different from investigation of the genes packed in the linear chromosomes, in terms of the size of the molecule and its haploid model of inheritance (Amorim et al., 2019). In addition, little to no noncoding DNA in mitochondrial genome renders higher probability of observing the change at the amino acid level with different functional consequences (Table 1). Additionally, physical proximity and occasional overlaps of the reading frames in mitochondrial genes lead to the protein-coding variant in one gene also being an upstream or downstream variant for another gene, thus potentially affecting the gene expression levels (Farge and Falkenberg, 2019). Mitochondrial diseases are rare, especially when compared to nuclear, but are presenting with severe phenotypes, early onset and high penetrance. Still, it is important to note that the variants we have observed, including those in the protein-coding region, did not generate any observable phenotypes in the participants.

Mitogenome sequencing in BiH has previously been confined to the control region analysis, in addition to RFLP genotyping of target coding sequence variants, with an aim of haplogroup assignment. While haplogroup assignment remains an important experimental approach, mainly for forensic and population genetics purposes, our team has started with Hypervariable Region 1 sequencing (Konjhodžić et al., 2023, 2024) with the goal to extend these efforts towards whole mitogenome sequencing and in-depth analysis for clinical purposes. It is necessary to have information on potential disease-associated mtDNA mutations and population trends in protein-coding mitochondrial gene variations. This study is the first step in this direction, offering pioneering results in clinical application of mtDNA sequencing, outside the haplotype-based migration studies in population genetics.

Considering the lack of relevant mtDNA-related studies in the population of Bosnia and Herzegovina (BiH), especially when it comes to the coding and clinically relevant sequence analysis, we aimed to undertake a pilot study in which the protein-coding genes of mtDNA will be sequenced for the first time in BIH, in order to support clinical use of mtDNA analysis healthcare, improve the mtDNA sequencing and genetic variant curation pipeline, and offer the first results on distribution of coding mtDNA sequence changes, as an upgrade to previous analyses of the noncoding sequence (Konjhodžić et al., 2023). This work represents our first preliminary results of *MT-ATP6* and *MT-ATP8* gene sequence analysis.

Conclusion

As the study primary contribution, we detected 11 different nucleotide variants in the *MT-ATP8* and *MT-ATP6* genes, three of which were previously unreported in the ClinVar reference database, while one is reported with uncertain significance. None of the identified mutations are pathogenic; two mutations have been identified as likely benign, but their disease association has yet to be determined. Five discovered SNPs were identified as benign mutations, most of which are associated with neuromuscular diseases such as Leigh syndrome, NARP syndrome, and Leber syndrome. The mutations identified in this study have a significant forensic influence on Bosnian-Herzegovinian population, especially due to their relatively low alternative allele frequencies, in addition to being adequate for usage in the guidelines for the routine clinical molecular diagnostics. This study provides input information

for the construction of a more elaborate study with an increased number of participants, so the results will be more beneficial and useful in forensic and clinical practice.

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Authors' contributions

N. Handžić:
Conceptualization; Funding acquisition; Methodology; Resources; Validation; Visualization; Writing – original draft; Writing – review & editing.

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L. Salihefendić:
Funding acquisition.

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Funding acquisition; Investigation; Supervision.

Conflict of interest

Authors declare no conflict of interest.

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