Received: 17 January 2022 | Accepted: 3 March 2022

DOI: 10.1002/jmv.27704

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Analysis of mitochondrial DNA cytochrome-b (*CYB*) and *ATPase-6* gene mutations in COVID-19 patients

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Abstract

Coronavirus disease of 2019 (COVID-19) is a pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Mutations of mitochondrial DNA (mtDNA) are becoming increasingly common in various diseases. This study aims to investigate mutations in the cytochrome-b (CYB) and adenosine triphosphatase-6 (ATPase-6) genes of mtDNA in COVID-19 patients. The association between mtDNA mutations and clinical outcomes is investigated here. In the present study, mutations of the mtDNA genes CYB and ATPase-6 were investigated in COVID-19 (+) ($n = 65$) and COVID-19 (−) patients ($n = 65$). First, we isolated DNA from the blood samples. After the PCR analyses, the mutations were defined using Sanger DNA sequencing. The age, creatinine, ferritin, and CRP levels of the COVID 19 (+) patients were higher than those of the COVID-19 (−) patients ($p = 0.0036$, $p = 0.0383$, $p = 0.0305$, $p < 0.0001$, respectively). We also found 16 different mutations in the CYB gene and 14 different mutations in the ATPase-6 gene. The incidences of CYB gene mutations A15326G, T15454C, and C15452A were higher in COVID-19 (+) patients than COVID-19 (−) patients; $p < 0.0001$: OR (95% CI): 4.966 (2.215−10.89), $p = 0.0226$, and $p = 0.0226$, respectively. In contrast, the incidences of A8860G and G9055A ATPase-6 gene mutations were higher in COVID-19 (+) patients than COVID-19 (−) patients; $p < 0.0001$: OR (95%CI): 5.333 (2.359−12.16) and $p = 0.0121$ respectively. Yet, no significant relationship was found between mtDNA mutations and patients' age and biochemical parameters ($p > 0.05$). The results showed that the frequency of mtDNA mutations in COVID-19 patients is quite high and it is important to investigate the association of these mutations with other genetic mechanisms in larger patient populations.

KEYWORDS

ATPase-6, COVID-19, CYB, mtDNA, PCR, Sanger sequencing

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; COVID-19, 2019 coronavirus; CRP, C-reactive protein; CYB, cytochrome-b; HCT, hematocrit; HGB, hemoglobin; LDH, lactate dehydrogenase; LYM, lymphocytes; MCV, mean corpuscular volume; mtDNA, mitochondrial DNA; NEU, neutrophils; PCR, polymerase chain reaction; PLT, platelets; WBCs, white blood cells.

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1 | INTRODUCTION

The coronavirus disease of 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a serious public health threat globally, endangering millions of people in a growing number of countries.[1] COVID-19 began in the Chinese city of Wuhan and has since extended to almost all countries of the world.[2] Many recent studies have described the epidemiological and clinical characteristics of symptomatic patients infected with SARS-CoV-2 remains largely unknown.[2] SARS-CoV-2 causes numerous cellular and systemic events that significantly impact the intracellular and extracellular mitochondrial activities and can lead to disease progression and severity.[3] Mitochondria play an essential role in the host's response to viral infection and immunity, which is the key to antiviral signaling and exacerbating inflammatory processes. Mitochondria have been identified as potential targets in SARS-CoV-2 infection.[4] The relationship between mitochondrial DNA (mtDNA) damage and COVID-19 infection is based on oxidative damage. mtDNA is vulnerable and exposed to oxidative stress as a result of metabolic function. In COVID-19 infection, increased reactive oxygen species (ROS) production can affect cell organelles including mitochondria.[5]

Mitochondria are crucial for cellular energy production.[6] mtDNA is a closed circular molecule that encodes 13 polypeptides which form oxidative phosphorylation complexes in humans.[7] mtDNA mutations and deletions are associated with oxidative stress, mitochondrial malfunction, and cell death.[7] mtDNA mutations can be occasional, genetic, or Mendelian in nature. Moreover, they can include mtDNA rearrangements such as deletions, inversions, or duplications, as well as point mutations.[8] mtDNA damage plays a key role in human aging, cancer, and neurological disorders. Point mutations of single bases or deletions of the 16.5-kb mitochondrial genome are the leading footprints of mtDNA damage.[9] Because the human mitochondrial genome is so tiny compared with the nuclear genome, mitochondrial genetics poses unique clinical and research questions.[10] Mitochondrial ATPase 6 (mt-ATP6) is a component of ATP synthase, a large enzyme that catalyzes the last stage of oxidative phosphorylation and is encoded by the mitochondrial genome.[11,12] mtDNA encodes three subunits of these complexes (I, III, and IV). The mitochondrial cytochrome-b (mt-CytB) gene encodes the mt-CytB protein, which is the only component of the respiratory complex III encoded by the mitochondrial genome that plays a key role in the electron transport system.[13]

Thus, the present study was performed to evaluate mitochondrial cytochrome-b (CYB) and ATPase-6 gene mutations in COVID-19-positive and -negative patients. We also investigated the association between these gene mutations and the clinical biochemical demographic features in COVID-19 patients.

2 | MATERIALS AND METHODS

2.1 | Collection of COVID-19-positive and -negative blood samples

COVID-19-positive ($n = 65$) and negative (control group) blood samples ($n = 65$) were included in this study. All blood samples were collected from individuals admitted to the Emergency Department of Ordu University Research Hospital with suspected COVID-19. Individuals with suspected COVID-19 and showing symptoms (e.g., cough, sore throat, shortness of breath, myalgia, fever, loss of taste, and chest pain) and who had undertaken polymerase chain reaction (PCR) tests were enrolled in the study. Blood samples were picked up randomly. The patients were separated into two groups according to whether the PCR test was positive or negative. Sampling was not conducted according to the severity of the disease. At the time of sample collection, vaccinations had not yet started in our country. Therefore, all individuals are unvaccinated. The necessary permissions for sample collection were obtained from the Turkish Republic's Ministry of Health and from Ordu University's Clinical Ethics Committee (number: 2021/26). All blood samples were collected in a hemogram tube (EDTA).

2.2 | Genomic DNA extraction from blood samples

The Eco→Tech DNA isolation kit (Cat no: EcoBGD-50x) was used to isolate genomic DNA from blood. For DNA isolation, 200 µl of blood was used for each sample and the isolation was successfully performed by following the protocol recommended by the manufacturer of the corresponding kit.

First, 200 µl of EcoSpin Lysis Buffer was added to each 200 µl whole-blood sample and mixed well. Then, 20 µl RNase A was added to the mixture from Step 1 and mixed well, which was incubated for 3 min at room temperature. Next, 20 µl Proteinase K was added to the mixture and mixed well, which was incubated for 10 min at 55°C. Then, 400 µl EcoSpin Binding Buffer was added and mixed well. After the washing and elution steps, the isolation was successfully completed.

The NanoDrop instrument Take3 Plate (BioTek) was used to measure the concentration of the DNA samples. The absorption ratios at 260 and 280 nm were used to evaluate the DNA purity. A ratio of approximately 1.8 is universally considered "pure" for DNA. All of the DNA samples were stored at −20°C before PCR.

2.3 | PCR and Sanger sequence analysis of CYB and ATPase-6 genes

The SensoQuest Labcycler device (thermalcycler) was used for the PCR stage. For each PCR reaction, 25 µl of EcoTaq 2× PCR Master Mix, 2 µl of forward primer (10 µM), 2 µl of reverse primer (10 µM), 10 pg −500 µg template DNA, and ddH_2O were used. Preoptimized primers

were preferred[14] (Table 1). PCR conditions were set as follows: 5 min at 95°C, 2 min at 94°C, 1 min at 61°C, 2 min at 72°C, 10 min at 72°C, and pause at 4°C for CYB and ATPase-6 genes. After PCR analysis, PCR products were run with 3 µl of ethidium bromide on a 2% gel. A 50 bp marker was used. Bands of 675 and 1064 bp amplified for CYB and ATPase-6 genes, respectively, were visualized in UV light.

The ABI3500 (Applied Biosciences) instrument was used for DNA sequencing. Before performing sequence analysis, the PCR products were cleared with exoSAP. After Sanger sequencing, analysis was performed using MITOMAP and Chromas Lite 2.1 (Technelysium) software.

2.4 | Identifying the variants of CYB and ATPase-6 genes by seven in silico programs

We used seven bioinformatics tools, Polymorphism Phenotyping v2 (PolyPhen-2), Protein Analysis Trough Evolutionary Relationship (PANTHER), Sorting Intolerant From Tolerant (SIFT), Protein Variation Effect Analyzer (PROVEAN), Mutation Assessor, Single Nucleotide Polymorphism Annotation Platform (SNAP) and Combined Annotation Dependent Depletion (CADD) to predict the functional effects of the variants of CYB and ATPase-6 gene.

2.5 | Hematological and biochemical tests

Routine test data of individuals admitted with suspected COVID-19 to the Emergency Department of Ordu University Research Hospital were used as hematological and biochemical parameters. In this study, parameters such as creatinine, white blood cells (WBCs), hemoglobin (HGB), platelets (PLT), neutrophils (NEU), lymphocytes (LYM), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), C-reactive protein (CRP), ferritin, blood urea nitrogen (BUN), hematocrit (HCT), and mean corpuscular volume (MCV) were evaluated. The means and standard deviations of test results obtained for the positive and negative samples were determined. We then performed statistical analyses by comparing the clinical data of the positive and negative samples.

2.6 | Statistical analysis

The program GraphPad Prism 7.04 was used for all of the statistical analyses. The normal distribution of the data was demonstrated by the Shapiro–Wilk normality test. We used mean ± SD to describe the normally distributed variables. The association between COVID-19 (+) and COVID-19 (−) patients and clinical parameters was examined using the Mann–Whitney *U* test. A χ^2 test was conducted to calculate the association between the mutation types. The association between mutations and clinical parameters was also examined using the Mann–Whitney *U* test. Values for $p < 0.05$ were accepted as statistically significant.

TABLE 1 Primer lists

CYB	Forward, 5'-TATCCGCCATCCCATACATT-3'
	Reverse, 5'-GGTGATTCCTAGGGGGTTGT-3
ATPase-6	Forward, 5'-AACGAAAATCTGTTCGCTTCAT-3'
	Reverse, 5'-ATGTGTTGTCGTGCAGGTAGAG-3'

3 | RESULTS

3.1 | COVID-19-positive and -negative individuals' clinical and demographic data distribution

The ages of our COVID-19 (+) patients ranged from 26 to 87 years, while those of COVID-19 (−) patients ranged in age from 18 to 92 years. In addition to COVID-19 disease, 52.31% (34/65) of our patients were also suffering from other diseases such as chronic obstructive pulmonary disease (COPD) (21.54%), cardiovascular disease (34.84%), hypertension (49.23%), diabetes (12.31%), chronic kidney disease (6.15%), neurological disease (12.31%), and hepatitis B (33.84%). Moreover, 18.47% (12/65) of our patients had other diseases, such as COPD (12.31%), cardiovascular disease (6.15%), hypertension (15.38%), diabetes (1.54%), chronic kidney disease (1.54%), neurological disease (4.62%), and hepatitis B (9.23%) in addition to COVID-19 (−) individuals.

The age, creatinine, ferritin, and CRP levels of COVID-19 (+) patients were higher than those of COVID-19 (−) patients ($p = 0.0036$, $p = 0.0383$, $p = 0.0305$, and $p < 0.0001$, respectively). However, there were higher levels of AST, ALT, LDH, WBC, PLT, NEU, LYM, BUN, HCT, and MCV in COVID-19 (+) patients than those in COVID-19 (−) patients ($p > 0.05$). Only HGB values were higher in COVID-19 (−) patients compared with COVID-19 (+) patients ($p > 0.05$). The age, hematological, and biochemical parameters of COVID-19 (+) and COVID-19 (−) patients are listed in Table 2.

3.2 | CYB and ATPase-6 gene PCR results

In all COVID-19 (+) and COVID-19 (−) patients included in the study, the mtDNA CYB and ATPase-6 genes were amplified by PCR. The 1064 bp (CYB) and 675 bp (ATPase-6) PCR products were analyzed in a 2% agarose gel (Figure 1A, B).

3.3 | CYB and ATP Sanger DNA sequence analysis results

The human mitochondrial genome sequence used to identify mutations was "Cambridge Reference Series" (http://www.mitomap.org) and analyzed using Chromas Lite software. The sequence analysis of the detected mutations is shown in Table 3. Sixteen

TABLE 2 Characteristics of COVID-19-positive patients and negative samples

Parameters (Reference range)	COVID-19 (+) patients (n = 65) (mean ± SD)	COVID-19 (−) samples (n = 65) (mean ± SD)	p
Age (years)	62.35 ± 19.71	51.58 ± 21.21	**0.0036
Creatinine (0.50–0.90 mg/dl)	1.11 ± 1.07	0.90 ± 0.68	*0.0383
AST (0–32 U/L)	23.94 ± 10.78	22.97 ± 14.62	0.3839
ALT (0–33 U/L)	23.8 ± 21.72	23.23 ± 13.73	0.6020
LDH (135–214 U/L)	238.06 ± 79.48	226.77 ± 86.47	0.3523
Ferritin (30–400 µg/L)	254.18 ± 281.08	130.50 ± 107.44	*0.0305
WBCs ($4.49–12.6 \times 10^3$/µl)	8.41 ± 5.79	7.94 ± 3.67	0.7620
HGB (12.3-15.3 g/dl)	12.58 ± 2.05	12.91 ± 2.05	0.2543
PLT ($150–450 \times 10^3$/µl)	240.88 ± 79.67	230.31 ± 89.65	0.6855
NEU ($1.8–6.98 \times 10^3$/µl)	5.80 ± 3.35	5.37 ± 3.34	0.3322
LYM ($1.26–3.35 \times 10^3$/µl)	1.96 ± 3.36	1.87 ± 1.37	0.1794
CRP (0–0.5 mg/dl)	5.18 ± 5.88	2.81 ± 6.01	****0.0001
BUN (6–18 mg/dl)	18.82 ± 14.78	16.06 ± 7.12	0.7882
HCT (%) (35.7–43.8)	38.47 ± 5.18	37.93 ± 5.834	0.8828
MCV (82.9–98 fl)	86.72 ± 6.59	86.23 ± 7.93	0.9434

Note: Mann–Whitney U test was used to calculate the association between the variables.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; COVID-19, Coronavirus disease of 2019; CRP, C-reactive protein; HCT, hematocrit; HGB, hemoglobin, LDH, lactate dehydrogenase; LYM, lymphocyte; MCV, mean corpuscular volume; NEU, neutrophils; PLT, platelets, WBCs, white blood cells.

*$p < 0.05$ is statistically significant.

different mutations were detected in the CYB gene and 14 different mutations were detected in the ATPase-6 gene in COVID-19 (+) patients. Seven different mutations (G15431A, T15747C, A15758G, C15452A, T15674C, A15326G, and T15693C) were missense type (nonsynonymous substitution), which causes amino acid alteration in the CYB gene. Six different mutations (G9055A, A8836G, T9070G, A8860G, A8701G, and G8950A) were missense type in the ATPase-6 gene. Eight mutations were synonymous substitution (not alter amino acids) type in both CYB (C15574T, T15310C, A15607G, G15301A, C15338T, T15454C, T15622C, and A15562G) and ATPase-6 gene (G8856A, A8901G, G8994A, G9123A, G8697A, C8943T, T8772C, and G8865A). In total, 90 missense mutations were determined in the patients.

3.4 | Distribution of CYB and ATPase-6 gene mutations in COVID-19 (+) patient and control groups

The mutations, nucleotide, and amino acid changes, mutation rates, and p values detected in the CYB and ATPase-6 genes are shown in Table 3. In addition, the rates of CYB and ATPase-6 mutations in the COVID-19 (+) and COVID-19 (−) patients are shown.

G15431A (n = 1), C15574T (n = 1), T15310C (n = 2), T15747C (n = 1), A15758G (n = 1), A15607G (n = 3), C15338T (n = 1), T15454C (n = 5), C15452A (n = 5), T15622C (n = 1), T15674C (n = 1), A15326G (n = 36), A15562G (n = 1), T15693C (n = 1), and T15804: frame shift (n = 1) mtDNA mutations were detected in the CYB gene in COVID-19 patients. In COVID-19 (−) patients, G15301A (n = 1) and A15326G (n = 13) mtDNA mutations were found in the CYB gene.

G8856A (n = 1), G9055A (n = 6), A8901G (n = 1), G8994A (n = 1), G9123A (n = 1), A8836G (n = 3), G8697A (n = 2), C8943T (n = 1), T9070G (n = 1), A8860G (n = 32), A8701G (n = 1), T8772C (n = 1), G8865A (n = 1), and G8950A (n = 1) mtDNA mutations were detected in the ATPase-6 gene in COVID-19 (+) patients. In the COVID-19 (−) patients, A8860G (n = 10) mtDNA mutations were found in the ATPase-6 gene.

COVID-19 (+) patients had significantly more A15326G, T15454C, and C15452A mutations in the CYB gene than COVID-19 (−) patients; $p < 0.0001$, OR (95% CI): 4.966 (2.215–10.89); $p = 0.0226$ and $p = 0.0226$, respectively. In contrast, A8860G and G9055A mutations of the ATPase-6 gene were more frequent in COVID-19 (+) patients than in COVID-19 (−) patients; $p < 0.0001$, OR (95%CI): 5.333 (2.359–12.16) and $p = 0.0121$; respectively) (Table 3).

A15326G and A8860G mutations were the most frequent mutation types in both the COVID-19 patient and control groups.

3.5 | Results of in silico analysis predicting the effects of human CYB and ATPase-6 gene variants

We used seven different in silico variants prediction tools, PolyPhen-2, PANTHER, SIFT, PROVEAN, Mutation Assessor, SNAP, and CADD, to predict the functional effects of the variants of CYB and ATPase-6 genes. As a result of the in silico analysis, we found that 13 missense types (nonsynonymous substitution) were found to have various effects on diseases (Table 4). G15431A and T15674C were predicted to be deleterious variants in the CYB gene by in silico programs. Moreover, G9055A, A8836G, and A8860G were predicted to be deleterious variants in the ATPase-6 gene.

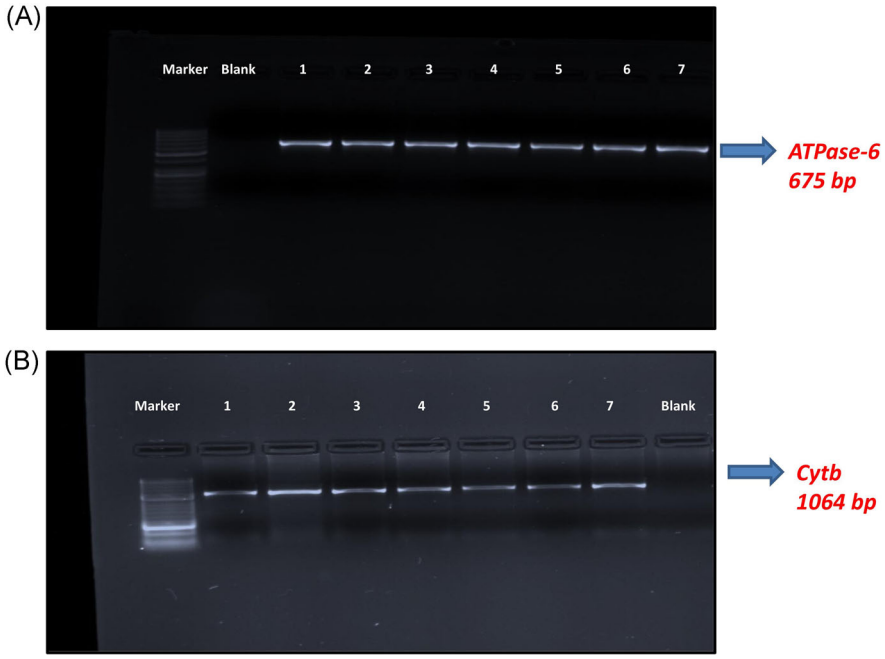

FIGURE 1 ATPase-6 and CYB gel electrophoresis image

3.6 | Clinical parameters in COVID-19 patients according to CYB and ATPase-6 gene mutational distribution

The age, hematological, and biochemical test results of the samples with and without (wild-type) CYB and ATP mutations are shown in Table 5. No significant relationship was found between the mtDNA mutations and patients' age, hematological, and biochemical parameters ($p > 0.05$). The mean age of patients with mutations (CYB and ATP) was lower than that of patients without mutations. The mean ALT, LDH, and MCV levels of patients with mutations (CYB and ATP) were higher than those of patients without mutations. The mean creatinine, ferritin, HGB, and PLT levels of patients with mutations (CYB and ATP) were lower than those of patients without mutations. Among the patients carrying the CYB gene mutation, 69.44% carried additional disease and among the patients carrying the ATPase-6 gene mutation, 65.63% carried additional disease.

4 | DISCUSSION

Mutations in the human mitochondrial genome are linked to several diseases, most of which are inherited from the mother and all of which are related to abnormalities in oxidative energy metabolism.[15] These diseases are currently incurable and virtually untreatable and have a wide range of penetrance, symptoms, and prognosis.[16] mtDNA mutations have been detected in many body fluids, including urine and saliva[17] and serum.[18] Mutations in OXPHOS mtDNA genes do not always result in alterations in the encoded protein.[19]

Viruses affect mitochondrial function, metabolism, and innate immune signaling.[20] Metabolism, calcium regulation, airway contractility in the lung, gene and protein balance, oxidative stress, and apoptosis are all affected by mitochondrial dysfunction. Mitochondrial dysfunction affects homeostatic cellular processes such as aging and senescence.[21] Mitochondria are proving to be significant in COVID-19 pathogenesis due to their function in innate antiviral immunity and inflammation.[22]

To the best of our knowledge, no previous research has investigated the association between CYB and ATPase-6 gene mutations in Turkish COVID-19 patients. In this study, the significance of CYB and ATPase-6 gene mutations in COVID-19 patients was investigated.

Clinical and laboratory findings were used to determine disease severity. The laboratory findings were not specific to COVID-19 infection; however, they were used to estimate patients' prognosis. Higher WBCs and NEU count, lymphopenia, thrombocytopenia, CRP, LDH, creatine kinase (CK), troponin, increased liver enzymes, impairment of coagulation mechanisms, and rinsed cytokines are related to the severity of COVID-19.[23,24] Some biomarkers, including C-reactive protein (CRP) and ferritin, have been reported to be useful in the literature.[23,25] Zhang et al.[25] reported that CRP level increased in COVID-19 patients. Moreover, with the increase in the CRP level in COVID-19, the development of ARDS and death can be observed.[26] During viral infections, the concentration of circulating ferritin rises and can indicate viral replication.[27] Ferritin levels have been reported to increase in tables where a cytokine storm is observed, such as COVID-19.[28] The laboratory findings of our patients showed that COVID-19 (+) patients had higher age, creatinine, ferritin, and CRP levels than COVID-19 (−) patients. Moreover, COVID-19 (+) patients had lower HGB values than COVID-19 (−) patients. In accordance with the literature, the laboratory findings vary according to disease severity.[29] Although the heterogeneity of the patients does not reflect the results confirmed by the literature, we can say that the laboratory findings were related to the severity of disease in our patients.

TABLE 3 Distribution of CYB and ATPase-6 mtDNA mutations in COVID-19-positive patients and negative samples

Gene	Nucleoid position	Nucleotide exchange	Amino acid change	Mutation type	Mutation rate (number of mutations/total number of patients)		
					Patient group	Control group	*p
CYB	15431	G → A	A229T	Missense	1/65	0/65	0.3154
CYB	15574	C → T	F276F	Transition	1/65	0/65	0.3154
CYB	15310	T → C	I188I	Transition	2/65	0/65	0.1541
CYB	15747	T →C	I334T	Missense	1/65	0/65	0.3154
CYB	15758	A → G	I338V	Missense	1/65	0/65	0.3154
CYB	15607	A → G	K287K	Transition	3/65	0/65	0.0797
CYB	15301	G → A	L185L	Transition	0/65	1/65	0.3154
CYB	15338	C → T	L198L	Transition	1/65	0/65	0.3154
CYB	15452	C → A	L236I	Missense	5/65	0/65	***0.0226**
CYB	15454	T → C	L236L	Transition	5/65	0/65	***0.0226**
CYB	15622	T → C	L292L	Transition	1/65	0/65	0.3154
CYB	15674	T → C	S310P	Missense	1/65	0/65	0.3154
CYB	15326	A → G	T194A	Missense	36/65	13/65	*****0.0001**
CYB	15562	A → G	W272W	Transition	1/65	0/65	0.3154
CYB	15804	T:	frmshft	Deletion	1/65	0/65	0.3154
CYB	15693	T → C	M316T	Missense	1/65	0/65	0.3154
ATPase-6	8856	G → A	A110A	Transition	1/65	0/65	0.3154
ATPase-6	9055	G → A	A177T	Missense	6/65	0/65	***0.0121**
ATPase-6	8901	A → G	L125L	Transition	1/65	0/65	0.3154
ATPase-6	8994	G → A	L156L	Transition	1/65	0/65	0.3154
ATPase-6	9123	G → A	L199L	Transition	1/65	0/65	0.3154
ATPase-6	8836	A → G	M104V	Missense	3/65	0/65	0.0797
ATPase-6	8697	G → A	M57M	Transition	2/65	0/65	0.1541
ATPase-6	8943	C → T	P139P	Transition	1/65	0/65	0.3154
ATPase-6	9070	T → G	S182A	Missense	1/65	0/65	0.3154
ATPase-6	8860	A → G	T112A	Missense	32/65	10/65	*****0.0001**
ATPase-6	8701	A → G	T59A	Missense	1/65	0/65	0.3154
ATPase-6	8772	T → C	T82T	Transition	1/65	0/65	0.3154
ATPase-6	8865	G → A	V113V	Transition	1/65	0/65	0.3154
ATPase-6	8950	G → A	V142I	Missense	1/65	0/65	0.3154

Note: χ^2 test was used to calculate the association between the variables.

*$p < 0.05$ is statistically significant.

More mutations occur in mtDNA than in nuclear DNA, and a correlation has been found between ROS increase and the age-related increase in mutant mtDNA.[30] Considering that SARS-CoV-2 indirectly produces the production of ROS, the cells of elderly people might be exposed to more ROS than those of healthy younger people when infected with this virus.[5] In our literature search, we did not find any articles addressing CYB and ATPase-6 variations in COVID-19 patients. However, mtDNA variations have been studied in many other diseases. According to the results of our Sanger analysis, 16 different mutations were found in the CYB gene and 14 different mutations were found in the ATPase-6 gene. Moreover, missense and synonymous substitutions were detected in CYB and ATPase-6 genes. The COVID-19 (+) patient group and the negative control group had the more common mt15326 A → G (in CYB) and

TABLE 4 Results of in silico analysis predicting the effects of human *CYB* and *ATPase-6* gene variants

Gene	Nucleoid position	Amino acid change	Polyphen2	PANTHER	SIFT	PROVEAN	Mutation assessor	SNAP	CADD
CYB	15431	A229T	Benign	Neutral	Neutral	Neutral	Low impact	Neutral	Deleterious
CYB	15747	I334T	Benign	Neutral	Neutral	Neutral	Low impact	Neutral	Neutral
CYB	15758	I338V	Benign	Neutral	Neutral	Neutral	Medium impact	Neutral	Neutral
CYB	15452	L236I	Benign	Neutral	Neutral	Neutral	Neutral impact	Neutral	Neutral
CYB	15674	S310P	Possible damaging	Disease	Neutral	Deleterious	High impact	Neutral	Deleterious
CYB	15326	T194A	Benign	Neutral	Neutral	Neutral	Neutral impact	Neutral	Neutral
CYB	15693	M316T	Benign	Neutral	Neutral	Neutral	Neutral impact	Disease	Neutral
ATPase-6	9055	A177T	Possible damaging	Disease	Neutral	Deleterious	Low impact	Neutral	Deleterious
ATPase-6	8836	M104V	Possible damaging	Disease	Neutral	Neutral	Low impact	Disease	Deleterious
ATPase-6	9070	S182A	Benign	Neutral	Neutral	Neutral	Neutral impact	Neutral	Neutral
ATPase-6	8860	T112A	Benign	Neutral	Neutral	Deleterious	Medium impact	Disease	Neutral
ATPase-6	8701	T59A	Benign	N/A	Neutral	Neutral	Neutral impact	Neutral	Neutral
ATPase-6	8950	V142I	Benign	Neutral	Neutral	Neutral	Neutral impact	Neutral	Neutral

mt8860 A → G (in ATPase-6) missense mutations. The COVID-19 (+) patient had significantly more A15326G, T15454C, and C15452A mutations in the CYB gene than the COVID-19 (−) patients. In addition, the COVID-19 (+) patients had higher frequency of A8860G and G9055A missense mutations of the ATPase-6 gene than in the COVID-19 (−) patients. However, no significant relationship was detected between CYB and ATPase-6 variants and the age and biochemical parameters of the patients. These results demonstrated that mtDNA is a part of cells that might be affected by COVID-19 infection. mtDNA mutations and clinical parameters can reflect disease severity more effectively if they are studied with an extended patient population.

Mitochondrial genetic changes have been proven to affect metabolic parameters and can play a role in bioenergetic pathways, metabolic rates, and energy consumption depending on the ethnic background.[31,32] Given the results of studies on other diseases associated with mtDNA variations, according to Mao et al.,[33] variations in the MT-ATP6 and MT-CYB genes might play a role in the unexpected fertilization disorder. The A8860G mutation in the ATPase-6 gene was detected in 79%–91.66% in breast tumors, 75%–100% in other types of cancers[32,33] and 92.85%–100% in neurodegenerative diseases.[34,35] In the present study, 32 COVID-19 (+) patients had A8860G missense mutation in the ATPase-6 gene. In another study, the frequencies of transversions in the ATP6 and CytB genes were found to be 96% and 97%, respectively, while the frequency of transversions in the ATP6 gene was found to be 4% and 3% in the CytB gene.[13] Pirola et al.[36] reported that NASH is associated with hereditary alterations in the cellular respirasome of the liver, including high cytochrome-b diversity and mtDNA damage, which can lead to widespread cellular effects. Li et al.[37] discovered three additional significant mitochondrial variants, mutations A4769G, A8860G, and A15326G, in samples taken from all 15 individuals in the family. They also assumed that these variants do not have much effect on mitochondrial work. In the current study, 36 COVID-19 patients had A15326G missense mutation in the CYB gene. G15431A and T15674C were predicted to be deleterious variants of the CYB gene by in silico programs. Moreover, G9055A, A8836G, and A8860G were predicted to be deleterious variants in the ATPase-6 gene by in silico programs. Our study indicates that these variants may change the secondary structure of the CYB and ATPase-6 proteins and subsequently can reduce their enzyme activity. The alteration of the enzyme activity can affect ROS and disease severity. In addition, these data suggest that more studies will need to be conducted to reveal the effects of these A8860G and A15326G mtDNA mutations in COVID-19 disease.

The current study has some limitations that should be mentioned. We examined CYB and ATPase-6 mutations and clinical parameters of patients with COVID-19. First, we were unable to analyze other mtDNA genes (ND1 and D310). Second, we did not have the opportunity to work with many patients. Therefore, additional research and study with larger patient populations are required to substantiate our findings and demonstrate their relevance.

To the best of our knowledge, there is no publication in the relevant literature that focuses on the association between CYB and ATPase-6 mutations and COVID-19 patients. As this is a pilot study, more data on mtDNA variants need to be collected to highlight the association between known mitochondrial variants and COVID-19 patients. The high prevalence of mtDNA mutations in COVID-19 patients suggests that they play a key role in the disease and alter patients' energy metabolism. We suggest that

TABLE 5 Characteristics of COVID-19-positive patients with *CYB* and *ATPase-6* gene mutation or wild type ($n = 65$)

Parameters(Reference range)	CYB (mean ± SD)			ATPase-6 (mean ± SD)		
	Mutant	Wild type	*p	Mutant	Wild type	*p
Age (years)	61.44 ± 22.09	63.48 ± 16.60	0.6819	60.16 ± 23.17	64.48 ± 15.72	0.7272
Creatinine (0.50–0.90 mg/dl)	0.98 ± 0.39	1.27 ± 1.54	0.7406	0.94 ± 0.29	1.27 ± 1.46	0.8680
AST (0–32 U/L)	24.61 ± 11.29	23.10 ± 10.25	0.6817	23.5 ± 8.97	24.36 ± 12.42	0.8576
ALT (0–33 U/L)	27.31 ± 27.37	19.45 ± 10.33	0.4403	24.44 ± 19.91	23.18 ± 23.64	0.7866
LDH (135–214 U/L)	241.39 ± 86.27	233.93 ± 71.43	0.7308	247.94 ± 88.26	228.49 ± 69.96	0.2975
Ferritin (30–400 µg/L)	237.92 ± 253.14	274.36 ± 315.80	0.9660	218.70 ± 189.05	288.60 ± 347.72	0.9196
WBCs ($4.49–12.6 \times 10^3$/µl)	8.91 ± 7.38	7.78 ± 2.84	0.9087	7.98 ± 3.12	8.83 ± 7.58	0.9351
HGB (12.3-15.3 g/dl)	12.51 ± 2.17	12.67 ± 1.93	0.8211	12.48 ± 2.16	12.68 ± 1.97	0.8170
PLT ($150–450 \times 10^3$/µl)	230.83 ± 64.61	253.35 ± 94.87	0.3845	239.97 ± 74.29	241.76 ± 85.71	0.9403
NEU ($1.8–6.98 \times 10^3$/µl)	6.00 ± 3.66	5.55 ± 2.96	0.8725	5.63 ± 2.90	5.96 ± 3.77	0.8121
LYM ($1.26–3.35 \times 10^3$/µl)	2.24 ± 4.45	1.60 ± 0.88	0.9765	1.63 ± 0.78	2.27 ± 4.66	0.6458
CRP (0–0.5 mg/dl)	5.52 ± 6.61	4.75 ± 4.92	0.7681	4.85 ± 5.77	5.49 ± 6.07	0.3783
BUN (6–18 mg/dl)	20.44 ± 16.70	20.04 ± 15.68	0.9347	19.25 ± 16.017	21.24 ± 16.42	0.4791
HCT (%) (35.7–43.8)	38.64 ± 5.72	38.51 ± 5.29	0.8467	38.36 ± 5.80	38.80 ± 5.25	0.9558
MCV (82.9–98 fl)	87.48 ± 6.23	85.37 ± 6.29	0.2078	87.44 ± 6.52	85.66 ± 6.04	0.3522

Note: Mann–Whitney *U* test was used to calculate the association between the variables.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; COVID-19, Coronavirus disease of 2019; CRP, C-reactive protein; HCT, hematocrit; HGB, hemoglobin, LDH, lactate dehydrogenase; LYM, lymphocyte; MCV, mean corpuscular volume; NEU, neutrophils; PLT, platelets, WBCs, white blood cells.

*$p < 0.05$ is statistically significant.

clinicians should consider the genetic background of patients when evaluating them.

AUTHOR CONTRIBUTIONS

Conceptualization, *methodology*, *software*, *validation*, *formal analysis*: Ebubekir Dirican, Gonca Gülbay, İsmail Erkan Aydın, Şeyda Tuba Savrun, Ülkü Karaman. *Investigation*, *resources*, *data curation*: Ebubekir Dirican, Gonca Gülbay, İsmail Erkan Aydın, Şeyda Tuba Savrun, Ülkü Karaman. *Writing—original draft*, *Writing—review and editing*: Ebubekir Dirican, Gonca Gülbay, İsmail Erkan Aydın, Şeyda Tuba Savrun, Ülkü Karaman. *Visualization*, *supervision*, *project administration*: Ebubekir Dirican, Gonca Gülbay, İsmail Erkan Aydın, Şeyda Tuba Savrun, Ülkü Karaman.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Dirican E, Savrun ŞT, Aydın İE, Gülbay G, Karaman Ü. Analysis of mitochondrial DNA cytochrome-b (*CYB*) and *ATPase-6* gene mutations in COVID-19 patients. *J Med Virol*. 2022;94:3138-3146. doi:10.1002/jmv.27704