Eurasian Journal of Molecular and Biochemical Sciences

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mtDNA damage, mtDNA mutations and Calpain 10 (CAPN10) gene expression status in type 2 diabetes patients

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Cite: *Dirican E, Kaya Y,. mtDNA damage, mtDNA mutations and Calpain 10 (CAPN10) gene expression status in type 2 diabetes patients. Eurasian Mol Biochem Sci 2023;2(1): 1-13.*

Received: 12 February 2023, Accepted: 07 March 2023

Abstract

In this study, we aimed to analyze mitochondrial DNA (mtDNA) mutations, mtDNA damage and expression level of the Calpain 10 (CAPN10) gene in patients with Type-2 diabetes (T2D). Whole blood was drawn from 45 healthy and 52 people with T2D for this investigation. For the analysis of mutations in the ATP6, ND1, CYB and D310 mtDNA genes, samples were first amplified by PCR. It was then confirmed by Sanger DNA sequencing. mtDNA -79 and mtDNA-230 fragments were amplified by RT-PCR to detect mtDNA damage. RT-PCR was also used for the mRNA expression of the CAPN10 gene. In patients with type-2 diabetes, m.8860 A→G (ATP6) (52/52), m.15326 A→G (CYB) (45/52), m.3384 A→T (ND1) (2/52) and m.489 T→C (D310) (11/52) were the most common mtDNA mutations. In addition, 7C (17/52), 8C (8/52) and 9C (1/52) mononucleotide repeats were detected in the D310 control region. An increase in the level of mtDNA-79, mtDNA-230 fragments and mtDNA integrity was determined in patients with T2Dcompared to healthy individuals (p=0.0090, p=0.9555, p=0.1213 respectively). The level of mtDNA-79 and mtDNA-230 fragments in patients showed a weak but significant positive correlation (p=0.0321, r=0.2977). In T2D patients compared to healthy people, the CAPN10 gene's mRNA expression was significantly higher (p=0.0360). Additionally, ROC analysis revealed that CAPN10 and mtDNA-79 had good diagnostic value in the patient group (AUC:0.603, p=0.0116, 95% CI:0.485-0.713, sensitivity: 53.8% for CAPN10 and AUC:0.653, p=0.0061, 95% CI:0.550-0.747, sensitivity: 51.9% for mtDNA-79). The findings showed that the frequency of mtDNA mutations and the expression level of the CAPN10 gene are high in patients with type-2 diabetes, and mtDNA damage is also increased. Keywords: Type-2 diabetes, CAPN10, mtDNA, mtDNA-79, mtDNA-230

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Introduction

Type 2 diabetes mellitus (T2DM) is known to be a critical important public health problem. Having a family history of diabetes significantly increases the risk of developing diabetes (1). Also, diabetes is causes a significant workload on the health system and increases the risk of death (2). T2DM is caused by genetic and environmental factors (3). T2DM is a disease associated with the dysregulation of metabolism, including insulin resistance, mitochondrial dysfunction, and oxidative stress (4). It

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is necessary to try to understand the molecular mechanisms that contribute to these metabolic disorders behind T2DM disease. Many factors contribute to insulin resistance, including a high-fat diet and physical inactivity, genetics, and lifestyle variables such as impaired mitochondrial function (5).

The mutation rate in mitochondrial DNA (mtDNA) is known to be 17 times higher than in nuclear DNA. mtDNA mutations may lead to a mitochondrial disease (6). These mutations are related to T2DM (7). Mitochondrial dysfunctions caused by mtDNA pathogenic mutations play putative roles in T2DM progression (8). However, how mitochondrial genome (mtDNA) abnormalities arise in patients with DM has not been fully elucidated (9). Patient-specific nonsynonymous mutations may be responsible together in identifying these diseases. The fact that some mtDNA variants are seen more frequently in patients than in controls may play a role in predisposing patients to these diseases (10). It has been shown that mtDNA mutations may cause to oxidative stress and impair mitochondrial function, which may play a role in the pathogenesis and progression of T2DM in this pedigree (11). Certain mitochondrial dynamics are significant for maintaining cellular metabolic homeostasis. Defects in some proteins may lead to loss of mtDNA integrity, disruption of mitochondrial function, drastic change in mitochondrial morphology, and eventual cell death (12). mtDNA damage may arise from increased levels of mitochondrial reactive oxygen species (mtROS) (13). Also, mtDNA mutations can be associated with higher ROS levels (14). Damage to the mtDNA impairs mitochondrial function, reduces ATP production, and changes the metabolite profiles (15). Cellular heteroplasmy caused by mtDNA damage results in loss of cell function and increased susceptibility to stress (16). Cells damaged by mechanical or infectious injury release fragments of proinflammatory mtDNA into the circulation (17). A measure of mtDNA integrity is the proportion of mtDNA-230 to mtDNA-79 fragments (18).

The CAPN10 gene is associated with T2DM. CAPN1, CAPN2 and CAPN10 members of the calpain system are involved in glucose metabolism (19). Cysteine protease CAPN10 is known to increase apoptosis and necrosis (20). CAPN1 and CAPN10 regulate glucose uptake in lymphocytes (21). CAPN10 is involved in insulin secretion, action and sensitivity to type 2 diabetes. The mechanism by which it affects insulin secretion and action has not been fully defined (22). CAPN10 can be expressed anywhere. It is also among the mitochondrial matrix proteases. It has been demonstrated that mitochondrial CAPN10 overexpression or knockdown causes cell death, indicating that mitochondrial CAPN10 is necessary for viability (23).

In the light of the above information, we aimed to determine mtDNA mutations, mtDNA damage and mRNA expression of the CAPN10 gene in patients with T2DM in this study. We also set out to show the distribution of these genetic changes according to the clinical and demographic data of the patients. This is first study to research these mechanisms together in T2DM patients, which can guide further studies.

Materials and Methods

Collection of patients with T2DM and healthy individuals: All samples were collected from individuals who were followed up at Ordu University Training and Research Hospital, Department of Internal Medicine. The Clinical Research Ethics Committee of the Atatürk University Faculty of Medicine approved this study (Ethics committee date: 30.06.2022, approval number: 2022/6-28). In this study, 52 patients with T2DM and 45 healthy individuals who did not have diabetes mellitus and additional comorbid diseases over the age of 18 were included in this study after taking each participant's informed consent. The height and weight of the

participants were assessed. Body Mass Index (BMI) was calculated [BMI = weight (kg)/height2 (meters)]. Fasting blood glucose, insulin, blood urea nitrogen (BUN), creatinine, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Triglyceride (TG), total cholesterol, HDL cholesterol, LDL cholesterol, HbA1c, Thyroid Stimulating Hormone (TSH), free t4 (FT4), sedimentation (ESR) and hemogram parameters were recorded from the patient files. Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index was calculated [HOMA-IR=Fasting Glucose(mg/dL) X Fasting Insulin(uIU/mL)/405]. Whole blood was collected from all individuals in a 5 cc EDTA tube.

Detection of mtDNA mutations and in silico analysis: DNA isolation stage was carried out in Bayburt University, SHMYO Medical Biology and Genetics laboratory. EcoTech DNA isolation kit (Erzurum, Turkey) was used for DNA isolation. 200 µl of whole blood was used for DNA isolation of each individual from whole blood taken from patients with T2DM. All isolation steps were carried out successfully according to the recommendations of the relevant company in the protocol (24, 25). DNA samples were archived in the refrigerator at -20oC until the PCR process was performed. After DNA isolation, the purity and concentration of DNA samples were measured using Take3 Plate (BioTek) spectrophotometry device at 260/280 nm wavelength. DNA samples were archived in the refrigerator at -20oC until the PCR process was performed.

All experiments in the PCR stage were conducted by Dr. Bayburt University, SHMYO Medical Biology and Genetics laboratory. For the analysis of ATP-6, CYB, ND1 and D310 mtDNA mutations, PCR was performed using the SensoQuest Labcycler (thermalcycler: heat cycler). For the PCR reaction, 25 µl of EcoTaq 2× PCR Master mix, 2 µl of forward and reverse primers (10 µM), 10 pg–500 µg template DNA and over with dH2O. The PCR program was adjusted according to the recommendations of the relevant company from which the master mix kit was purchased (24, 25). mtDNA gene primers were taken from a previous study (26) (Table 1).

Table 1. Primer pairs

mtDNA-230	Forward: 5'- AGCCGCTATTAAAGGTTCG -3' Reverse: 5'- GGGCTCTGCCATCTTAACAA -3'
mtDNA-79	Forward: 5'- AGCCGCTATTAAAGGTTCG-3' Reverse: 5'- CCTGGATTACTCCGGTCTGA-3'
GAPDH	Forward:5′- CCCCACACACATGCACTTACC-3′ Reverse: 5′- CCTAGTCCCAGGGCTTTGATT-3
ATP-6	Forward: 5′- AACGAAAATCTGTTCGCTTCAT-3′ Reverse: 5′- ATGTGTTGTCGTGCAGGTAGAG-3′
ND1	Forward: 5'- CCAACCTCCTACTCCTCATTGT-3' Reverse: 5'- TGATCAGGGTGAGCATCAAA-3'
D310	Forward: 5'-ACAATTGAATGTCTGCACAGCCACTT-3′ Reverse: 5'- GGCAGAGATGTGTTTAAGTGCTG-3′
CYB	Forward: 5'- TATCCGCCATCCCATACATT-3′ Reverse: 5'-GGTGATTCCTAGGGGGTTGT-3′
Calpain-10	Forward: 5′ CACCTACCTGCCGGACACA-3′ Reverse:5′- TGCCATGACGGAGACCTCTT-3′
β-actin	Forward:5′-TCACCCACACTGTGCCCATCTACGA-3′ Reverse: 5′-CAGCGGAACCGCTCATTGCCAATGG-3′

After the PCR step, run on a 2% agarose gel using 3 µl of ethidium bromide and targeted 1064 bp bands were confirmed in the Bio-Vision UV device using a 50 bp marker. Then, PCR purification was performed with ExoSAP, and Sanger DNA sequence analysis was performed using the ABI PRISM 310 device as service procurement. Sanger DNA sequence analysis results were evaluated using Unipro UGENE v43.0 program. Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/), Mutation Assessor (http://mutationassessor.org/r3/), Protein Analysisthrough Evolutionary Relationships (Panther) (http://www.pantherdb.org/), Combined Annotation-Dependent Depletion (CADD) (https://cadd.gs.washington.edu/), Protein Variation Effect Analyzer (PROVEAN) (https://www.jcvi.org/research/provean) and Sequence homology-based results from Sorting Intolerant from Tolerant (SIFT) (https://sift.bii.a-

star.edu.sg/) six different in silico analysis databases were used for in silico analysis of mtDNA mutations.

Analysis of mtDNA damage: Primers of mtDNA-79, mtDNA-230 and GAPDH that had been used in a previous study were purchased (27, 28) (Table 1). A DNA fragment of 79 base pairs (bp) was amplified by the primer pair for mtDNA-79 and a fragment of 230 bp by the primer pair for mtDNA-230. Mitochondrial fragmentation status was defined as mtDNA integrity by calculating the relative ratio of mtDNA-230 to mtDNA-79. GAPDH DNA was used to normalize the PCR data in our study. iTaq Universal SYBR Green supermix (2X) was used and the reaction was started by adjusting the PCR program according to the kit's recommendations.

The PCR conditions were 95°C for 2-5 min, followed by 35-40 cycles at 95°C for 2-5 sec and 60°C for 15-30 sec. Using the $2^{-\Delta\Delta ct}$ method, the relative amounts of mtDNA fragments were calculated.

mRNA expression analysis of the CAPN10 gene: RNA isolation and cDNA synthesis steps were carried out in Bayburt University, SHMYO Medical Biology and Genetics laboratory. EcoPURE Total RNA isolation kit was used for RNA isolation. 100 µl of whole blood was used. RNA isolation of healthy and sick individuals was successfully performed according to the recommendations in the protocol of the kit (25, 29). At the last of isolation stage, 50-100 µl of EcoPURE Elution Buffer was added to the filter and incubated for one minute. In the next step, centrifugation was carried out for 30 seconds at maximum speed. All RNA samples were archived in the refrigerator at -20°C. The cDNA reaction was performed with the iScript cDNA synthesis kit. The cDNA synthesis process was performed using the SensoQuest Labcycler (thermalcycler: heat cycler) device according to the recommendations in the protocol of the kit (25,29).

Gene expression analyses were performed with the Bio-Rad CFX-96 RT-PCR device. For the mRNA expression reaction, samples were prepared for the reaction with 10 µl of iTaq Universal SYBR Green supermix (2X), 5 µl of cDNA, 1 µl of primers (CAPN10 and beta-actin) (30) (Table 1), and 3 µl of PCR-grade water. The recommended RT-PCR program in the master mix protocol was adjusted and the reaction was run (25). The relative mRNA expression of CAPN10 was analyzed by the $2^{-\Delta\Delta ct}$ method.

Statistical analyses: The acquired results were statistically analyzed using the programs GraphPad Prism 9.4.0 and MedCalc version 18.11.6. For normalization, the Kolmogorov-Smirnov (K-S) was used. The To compare the biochemical and demographic information of patients with T2DM and healthy individuals, Mann-Whitney U and Student's t-test were used. The levels of Calpain 10, mtDNA-79, 230 and integrity were compared between the patient and healthy group using the Mann-Whitney U test.

Spearman correlation test was used for correlation analysis of mtDNA-79 and 230 mtDNA damage markers in healthy and patient groups. The diagnostic potency and significance of the mtDNA damage markers CAPN10, mtDNA-79 and 230 were demonstrated using ROC curve analysis. The Kruskal-Wallis test was employed to distribution of CAPN10, mtDNA-79 and 230 levels according to the mtDNA mutations with the highest frequency. The distribution of CAPN10, mtDNA-79, and 230 levels in relation to demographic and biochemical data was displayed using the Mann-Whitney U test. Display the distribution of CAPN10, mtDNA-79, and 230 levels in relation to 7C and 8C mononucleotides in the D310 control region, the Mann-Whitney U test was employed. If $p< 0.05$, statistical results were deemed significant.

Results

Characteristics of patients with T2DM and healthy individuals: Table 2 summarizes the clinical and demographic information of patients with T2DM. There were 25% men and 75% women among patients with T2DM. The patients' mean age was 44.94 with a

standard deviation of 5.53. 87% of healthy individuals were female and 13% were male. Their mean age was 43.35±10.70 (mean±sd). T2DM patients had statistically significant differences in BMI, fasting blood glucose, ALT, TG, HDL cholesterol and HbA1c levels compared to healthy individuals (p<0.0001; p<0.0001; p=0.018; p=0.0013; p=0.0014; p<0.0001, respectively).

Table 2. Distribution of demographic and various biochemical data of individuals

Data	healthy individuals (n=45)	patient individuals (n=52)	*P* value
	mean±sd	mean±sd	
WBC[1] (4.49-12.58x10^9/L)	6.28±1.86	7.26±1.92	0.4902
HDL cholesterol[1] (45-65 mg/dL)	58.79±14.42	49.75±12.20	**0.0014
LDL cholesterol[1] (0-130 mg/dL)	108.23±29.41	103.91±27.45	0.7014
BMI[2] (19 - 24.9 kg/m^2)	29.19±3.90	34.45±5.903	****<0.0001
Total cholesterol[2] (0-200 mg/dL)	187.88±34.72	190.7±53.83	0.5386
FT4[1] (0.93-1.70 ng/dL)	1.22±0.27	1.49±0.41	0.135
PLT[1] (150-450x10^9 /L)	261842.1±83849.91	265000±104177.5	0.4289
AST[1] (0-32 U/L)	16.16±3.17	21.31±15.56	0.1395
ALT[1] (0-33 U/L)	15.32±4.82	23.95±14.21	**0.0018
CRE[1] (0.50-0.90 mg/dL)	0.65±0.12	0.76±0.22	0.461
TSH[1] (0-200 mlU/L)	1.75±0.98	2.06±1.82	0.4723
Insulin[1] (2.6-24.9 mlU/L)	10.29±4.84	16.67±8.26	0.0889
BUN[1] (0-18 mg/dL)	10.72±2.46	15.22±4.38	0.2019
HbA1C[1] (0-6.5 mg/dL)	5.58±0.43	7.71±1.63	****<0.0001
ESR[1] (0-15 mm/h)	21.39±7.56	29.10±13.70	0.0768
HB[1] (11.9-14.6 g/dL)	12.36±1.57	12.5±2.01	0.2108
TG[1] (0-200 mg/dL)	126.77±71.52	147.7±47.38	**0.0013
Fasting blood sugar [1] (70-100 mg/dL)	95.5±11.06	154.35±42.84	****<0.0001
HOMA-IR[1] (index, 0-2.5)	2.41±1.27	3.975±1.60	0.0533
Age (years) [1]	43.35±10.70	44.94±5.53	0.9145

[1] Mann-Whitney U test was used. [2] Student's t-test was used. sd: Standard deviation *p<0.05 was considered statistically significant (*: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001)

Distribution of mtDNA mutations in patients with T2DM and in silico analysis: A total of 182 mutations from 14 different ATP6, 13 different CYB, four different ND1 and five different D310 gene mutation types were identified in T2DM patients. Two different mutation types were identified in 33 patients, three different mutation types in four patients, four different mutation types in 3 patients and five different mutation types in one patient (Table 3).

The state of patients with T2DM carrying ATP6, CYB, ND1 and D310 (control region) mtDNA mutations was demonstrated by Sanger DNA sequencing analysis (Figure 1). Missense mutation was detected A→G at nucleotide position 8860 (ATP6 gene) in 52 of the patients. Other ATP6 mutations are C→T at position 8684 (n=1), T→C at position 8772 (n=1), C→T at nucleotide position 8818 (n=2), G→A at position 8950 (n=1), T→C at nucleotide position 8966 (n=2), G→A at position 8994 (n=1), G→A at nucleotide position 9055 (n=1), T→C at nucleotide 9149 (n=1), C→A at nucleotide position 9154 (n=2), T→A at position 9164 (n=2), T→A at nucleotide 9165 (n=1), T→A nucleotide 9166 (n=2), at position 9167 and T→A mutations at nucleotide position 9167 (n=2) were identified.

Table 3. Patients with type 2 diabetes's mtDNA mutation prevalence

Gene	Nucleotide position	Nucleotide exchange	Amino acid exchange	Mutation type	Grantham value	Mutation rate (number of mutations/Total number of patients)
ATP6	8684	C→T	Thr53Ile	Missense	89	1/52
ATP6	8772	T→C	Thr82=	Synonymous	0	1/52
ATP6	8818	C→T	Leu98=	Synonymous	0	2/52
ATP6	8860	A→G	Thr112Ala	Missense	58	52/52
ATP6	8950	G→A	Val142Ile	Missense	29	1/52
ATP6	8966	T→C	Ile147Thr	Missense	-	2/52
ATP6	8994	G→A	Leu156=	Synonymous	0	1/52
ATP6	9055	G→A	Ala177Thr	Missense	58	1/52
ATP6	9149	T→C	Leu208Ser	Missense	-	1/52
ATP6	9154	C→A	Gln210Lys	Missense	-	2/52
ATP6	9164	T→A	Val213Asp	Missense	-	2/52
ATP6	9165	T→A	Val213=	Synonymous	0	1/52
ATP6	9166	T→A	Phe214Ile	Missense	-	2/52
ATP6	9167	T→A	Phe214Tyr	Missense	-	2/52
CYB	14798	T→C	Phe18Leu	Missense	22	2/52
CYB	14831	G→A	Ala29Thr	Missense	58	1/52
CYB	14905	G→A	Met53Ile	Missense	0	1/52
CYB	15043	G→A	Gly99=	Synonymous	0	7/52
CYB	15148	G→A	Pro134=	Synonymous	0	1/52
CYB	15204	T→C	Ile153Thr	Missense	89	1/52
CYB	15218	A→G	Thr158Ala	Missense	58	1/52
CYB	15301	G→A	Leu185=	Synonymous	0	16/52
CYB	15310	T→C	Ile188=	Synonymous	-	1/52
CYB	15326	A→G	Thr194Ala	Missense	58	45/52
CYB	15452	C→A	Leu236Ile	Missense	5	4/52
CYB	15487	A→T	Pro247=	Synonymous	0	1/52
CYB	15607	A→G	Lys287=	Synonymous	0	1/52
ND1	3384	A→T	Lys26Asn	Missense	0	2/52
ND1	3591	G→A	Leu95=	Synonymous	0	1/52
ND1	3663	A→G	Ser119=	Synonymous	0	1/52
ND1	3834	G→A	Leu176=	Synonymous	0	1/52
D310	456	C→T	Control Region	Non-coding	-	2/52
D310	462	C→T	Control Region	Non-coding	-	4/52
D310	489	T→C	Control Region	Non-coding	-	11/52
D310	497	C→T	Control Region	Non-coding	-	1/52
D310	499	G→A	Control Region	Non-coding	-	4/52
Total number of mutations detected in all patients:						182
Number of patients carrying 1 mutation:						87
Number of patients carrying 2 mutations:						33
Number of patients carrying 3 mutations:						4
Number of patients carrying 4 mutations:						3
Number of patients carrying 5 mutations:						1
Alterations in the D310 mononucleotide repeat						Mutation rate (number of mutations/Total number of patients)
7C (normal)						17/52
8C						8/52
9C						1/52
Total number of mutations detected in all patients:						26

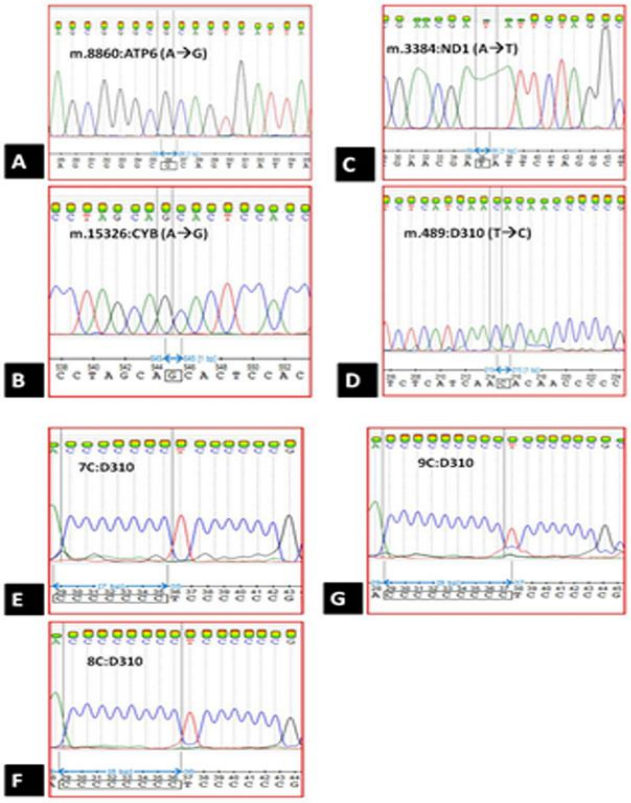

Figure 1: Sanger DNA sequence analysis results of mtDNA mutations. A. m.8860: ATP6 (A→G), B. m.15326: CYB (A→G), C. m.3384: ND1 (A→T), D. m.489:D310 (T→C), E. 7C:D310, F. 8C:D310, G. 9C:D310

Missense mutation was detected A→G at nucleotide position 15326 (CYB gene) in 45 of the patients. Other CYB mutations are T→C at position 14798 (n=2), G→A at position 14831 (n=1), G→A at position 14905 (n=1), G→A at nucleotide position 15043 (n=7), G→A at position 15148 (n=1), T→C at nucleotide position 15204 (n=1), A→G at position 15218 (n=1), G→A at nucleotide position 15301 (n=16), T→C at nucleotide 15310 (n=1), C→A at nucleotide position 15452 (n=4), A→T at position 15487 (n=1) and A→G at nucleotide 15607 (n=1) were identified.

Missense mutation was detected A→T at nucleotide position 3384 (ND1 gene) in 2 of the patients. Other ND1 mutations are G→A at position 3591 (n=1), A→G at position 3663 (n=1) and G→A at position 3834 (n=1) were identified.

T→C nucleotide change was detected at position 489 in the D310 control region in 11 of the patients. Other D310 mutations are C→T at position 456 (n=2), C→T at position 462 (n=4) and C→T at position 497 (n=1) and G→A at position 499 (n=4) were identified.

Patients with T2DM were the most detected 7C (n=17) and 8C (n=8) variants in D310 control region. Other D310 variant is 9C (n=1). One patient had a 9C variant. A score called the Grantham score (value) defines the difference in side chain atomic composition, polarity, and volume between two amino acids (31). Grantham score conserved (0-50), moderately conserved (51-100), moderately radical (101- 150) or radical (≥151) (32). When we investigated the Grantham scores of mutations in the ATP6 gene were 89 for m.8684, 58 for m.8860, 29 for m.8950 and 58 for m.9055. The Grantham scores of mutations in the CYB gene; 22 for m.14798, 58 for m.14831, 89 for m.15204, 5 for m.15452, 58 for m.15218 and m.15326. However, the Grantham score of all the mutations we identified was not defined (Table 3). Some of the mutations we identified had a Grantham score of zero (0) (m.8772, m.8818, m.8994, m.9165, m.14905, m.15043, m.15148, m.15301, m.15487, m.15607, m.3384, m.3591, m.3663 and m.3834).

In this study, six different in silico analysis databases, namely PolyPhen-2, Mutation Assessor, Panther, CADD, PROVEAN, and SIFT were used for in silico analysis of mtDNA mutations. According to the results of in silico analysis, m.8684, m.8860, m.8950, m.8966, m.9055, m. 9149, m.9154, m.9164, m.9166, m.9167, m.14798, m. 14831, m.15204, m.15218, m.15326, m.15452 and m.3384 mutations were found to have benign, disease-causing or harmful effects in some diseases (Table 4).

Level of mtDNA damage in T2DM and healthy individuals: It was demonstrated that patients with T2DM had higher levels of the mtDNA-79, 230 fragments and mtDNA integrity levels than did healthy people (p=0.0090, p=0.955, p=0.1213 respectively)

(Figures 2A-C). In addition, when mtDNA-79 and mtDNA-230 were found to increase relatively to healthy individuals with T2DM, a weak and significant positive correlation was shown between mtDNA-79 and mtDNA-230 fragments when analyzing whether there was any correlation between them (p=0.0321, r=0.2977). On the other hand, when mtDNA-79 and mtDNA-230 fragments were analyzed for correlation in healthy persons, a moderate and substantial positive correlation (p=0.0019, r=0.4496) was found. When we grouped them according to the mtDNA-79 level and the high and low TSH levels, a difference was determined between the data (p=0.0159). However, mtDNA-79 and mtDNA-230 levels did not significantly differ when compared to demographic data. (p>0.05).

According to the ROC analysis results, the AUC value of mtDNA-79 was determined as 0.653 and the confidence interval (CI: 95% CI) (0.550-0.747) (p=0.0061). The AUC value of mtDNA-230 was determined as 0.503 and the confidence interval (CI: 95% CI) (0.400-0.607) (p=0.955). While the mtDNA-79 gene has an important diagnostic value with 51.9% sensitivity and 73.3% selectivity for patients with T2DM and the mtDNA-230 gene has been shown to have an important diagnostic value with 19.2% sensitivity and 97.8% selectivity (Figures 3A-B).

Table 4. *In silico* analysis results of mtDNA mutations

Gene	Nucleotide position	PolyPhen2	Mutation Assessor	PANTHER	CADD	SIFT	PROVEAN
ATP6	8684 (C→T)	Benign	Neutral impact	Neutral	Neutral	Neutral	Neutral
ATP6	8860 (A→G)	Benign	Medium impact	Neutral	Neutral	Neutral	Deleterious
ATP6	8950 (G→A)	Benign	Neutral impact	Neutral	Neutral	Neutral	Neutral
ATP6	8966 (T→C)	Benign	Medium impact	Disease	Deleterious	Neutral	Deleterious
ATP6	9055 (G→)	Possibly damaging	Low impact	Disease	Deleterious	Neutral	Deleterious
ATP6	9149 (T→C)	Probably damaging	Medium impact	-	Deleterious	Neutral	Deleterious
ATP6	9154 (C→A)	Probably damaging	High impact	-	Deleterious	Deleterious	Deleterious
ATP6	9164 (T→A)	Probably damaging	High impact	-	Deleterious	Deleterious	Deleterious
ATP6	9166 (T→A)	Probably damaging	High impact	-	Deleterious	Neutral	Deleterious
ATP6	9167 (T→A)	Probably damaging	High impact	-	Deleterious	Deleterious	Deleterious
CYB	14798 (T→C)	Benign	Neutral impact	Neutral	Neutral	Neutral	Neutral
CYB	14831 (G→A)	Benign	Low impact	Neutral	Neutral	Neutral	Neutral
CYB	15204 (T→C)	Benign	Medium impact	Disease	Neutral	Neutral	Deleterious
CYB	15218 (A→G)	Probably damaging	Medium impact	-	Neutral	Neutral	Neutral
CYB	15326 (A→)	Benign	Neutral impact	Neutral	Neutral	Neutral	Neutral
CYB	15452 (C→A)	Benign	Neutral impact	Neutral	Neutral	Neutral	Neutral
ND1	3384 (A→T)	Probably damaging	High impact	-	Deleterious	Neutral	Deleterious

Polymorphism Phenotyping v2 (PolyPhen- 2) (http://genetics.bwh.harvard.edu/pph2/),

Mutation Assessor (http://mutationassessor.org/r3/),

Protein Analysis Through Evolutionary Relationships (PANTHER) (http://www.pantherdb.org/),

Combined Annotation-Dependent Depletion (CADD) (https://cadd.gs.washington.edu/),

Protein Variation Effect Analyzer (PROVEAN) (https://www.jcvi.org/research/provean)

Sequence homology-based results from Sorting Intolerant from Tolerant (SIFT) (https://sift.bii.a-star.edu.sg/)

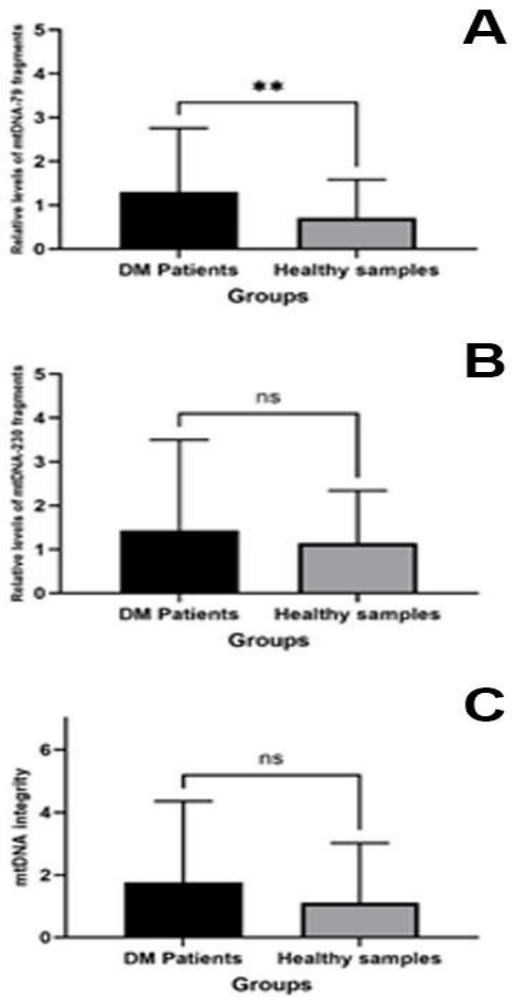

Figure 2: A. mtDNA-79 levels in T2DM and healthy individuals (**p=0.0090) B. mtDNA-230 levels in T2DM and healthy individuals (p=0.955) C. mtDNA integrity levels in T2DM and healthy individuals (p=0.1213)

Calpain-10 mRNA expression in T2DM and healthy individuals: The CAPN10 gene's mRNA expression level was higher in patients with T2DM patients than it was in healthy people (p=0.0360) (Figure 4). The ROC analysis results, the AUC value of CAPN10 was determined as 0.603 and the confidence interval (CI: 95% CI) (0.485-0.713) (p=0.116). CAPN10 gene has been shown to have an important diagnostic value with 53.8% sensitivity and 68.4% selectivity (Figure 3C).

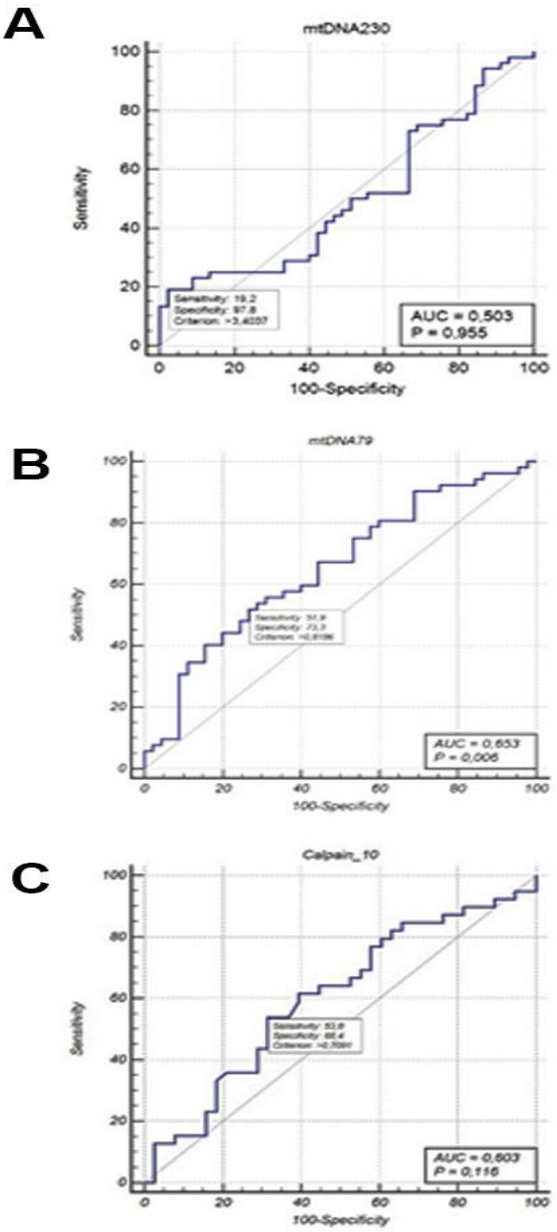

Figure 3: A. ROC analysis result of mtDNA-230, B. ROC analysis result of mtDNA-79, C. ROC analysis result of Calpain-10

Distributions of the Calpain-10 mRNA expression and mtDNA markers in individuals carrying mtDNA mutations in patients with T2DM: In individuals with mtDNA mutations, mtDNA-79, mtDNA-230 and CAPN10 gene expression levels were not statistically different according to the type of mutation (p>0.05). The mutation type with the highest expression of CAPN10 was determined as m.15326 and the mutation type with the highest mtDNA-79 and mtDNA-230 was determined as m.462.

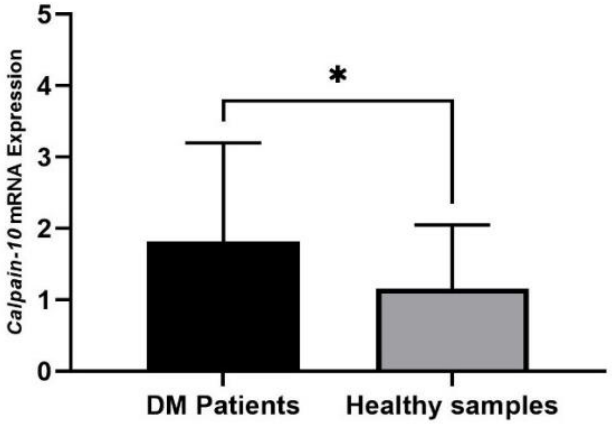

Figure 4: Expression status of CAPN10 gene in T2DM and healthy individuals (*p=0.0360)

Discussion

The prevalence of T2DM continues to rise globally (33). In the onset and progression of T2DM, genetic and environmental factors are significant contributors. In this study, we screened mtDNA variants in patients with T2DM. We determined the level of the mtDNA damage markers, mtDNA-79 and mtDNA-230, and the CAPN10 gene expression, which has been shown to be important in patients with T2DM.

Mitochondrial dysfunction has been shown to be related with T2DM (34). The mtDNA 3243A > G gene mutation has been reported that diabetes mellitus with mtDNA 3243(A-G) mutation represents 0.5-2.8% of the general diabetic population (35). Patients with type T2DM have the m.9053G>A mutation in the ATP6 gene. The ATP6 gene is a crucial component of ATP synthase for proton and electron transport in the internal respiratory system (36). A C15735T missense mutation in the CYB gene in T2D was detected (10). Maternally inherited diabetes and deafness have been linked to mutations in the mitochondrial 12S rRNA gene (m.1555A>G) and the ND1 gene (m.3308T>C) (37).

In this study, 182 mutations from 14 different ATP6, 13 different CYB, four different ND1 and five different D310 gene mutation types were determined in T2DM patients. In addition, two different mutation types were found in 33 patients, three different mutation types in four patients and five different mutation types in one patient. In patients with T2DM, m.8860 A→G (ATP6) (52/52), m.15326 A→G (CYB) (45/52), m.3384 A→T (ND1) (2/52) and m.489 T→C (D310) (11/52) were the most common mtDNA mutations. In addition, 7C (17/52), 8C (8/52) and 9C (1/52) mononucleotide repeats were detected in the D310 control region.

The Grantham score is the calculation of the evolutionary distance between two amino acids. A low Grantham score is known to reflect less evolutionary distance (31). In T2DM patients the highest Grantham score was determined as 89 for m.8684 (ATP6) and m.15204 (CYB), 58 for m.8860 (ATP6), m.9055 (ATP6), m.15218 (CYB) and m.15326 (CYB).

For the in silico analysis of mtDNA mutations in the current study, six different in silico analysis databases were used. mtDNA mutations identified in patients with T2DM have been demonstrated to have benign, pathogenic, or deleterious consequences in several disorders, according to the finding of in silico analysis.

Age-related disorders like T2DM have an etiology that is significantly influenced by the accumulation of oxidative damage in DNA and other macromolecules (38). One redox disorder is T2DM. Glucose intolerance results from altered metabolic homeostatic set points caused by oxidative damage and chronic inflammation (39). mtDNA is particularly sensitive to oxidative stress. If mtDNA damage accumulates, the resulting mitochondrial dysfunction disrupts normal cellular function and may cause a bioenergy crisis that accelerates aging and related diseases (40). Mitochondrial dysfunction and mtDNA variations can be seen during insulin resistance. These changes may be related to a difference in ATP levels in events such as ROS production, mitochondrial division/fusion, and mitophagy (41). Damaged mtDNA may be involved in the regulating of hyperglycemia during T2DM (42). Adipocytes' mitochondrial malfunction may affect adipogenesis, insulin sensitivity, obesity, and T2DM

(43). mtDNA integrity is evaluated the ratio of mtDNA-230/79 fragments. In addition, DNA integrity measured in blood indicates cell death (28). To our knowledge, there has been no previous study that looked at mtDNA fragments in T2DM patients. According to this study, patients with T2DM have higher levels of the mtDNA-79, 230 fragments and integrity than healthy people.

CAPN10 is an unusual calpain that is expressed all over the body and is localized in the cytosol, mitochondria, and nucleus (44). Insulin secretion and action involve CAPN10 (45). Because CAPN10 is produced in several organs, the protein's target substrates and additional relevant variables may interact to induce diabetes (46). One study evaluated the mRNA and protein levels of CAPN1, CAPN2, and CAPN10. No significant changes in mRNA levels were found in the expression of any of the calpains tested (19). Calpain proteases can be affected by their cellular location or post-translational changes (47, 48). It was also emphasized that further studies are needed to show whether CAPN2 or CAPN10 is deregulated in leukocytes during T2D development (19). CAPN10 mRNA was found to be more abundant in the pancreatic islets of patients with T2D (49). In this study, we found the mRNA expression of CAPN10 to be higher in the T2DM patient group compared to healthy individuals. In addition, there was a difference in CAPN10 level in the patient group according to TSH level. According to other demographics, patient clinical information, and mtDNA mutation type, there was no change in the level of CAPN10.

This study had several limitations. One of them is that it has not received any project support and therefore, the number of patients is low. The other is that both other genes of mtDNA and other Calpains have not been studied. In the future, it will be valuable to look at these genetic mechanisms both in blood and serum/plasma. Moreover, we plan to conduct new research to fill these gaps in the literature and we think that this study will be a guide for other researchers.

In conclusion, in this study, it was shown that mtDNA variants were at high frequency in patients with T2DM, and that mtDNA damage biomarkers were also at high levels. Calpain-10 gene mRNA expression was also found to be high in the patient group. The mutation type with the highest expression of CAPN10 was determined as m.15326, and the mutation type with the highest mtDNA-79 and mtDNA-230 was determined as m.462. To our knowledge, this is valuable as it is the first study to investigate at these genetic changes with T2DM. It may be helpful for the diagnosis, treatment, or prognosis of the disease to investigate these genetic mechanisms in a wider patient group.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions: Concept/Design: ED, YK; Data acquisition: ED, YK; Data analysis and interpretation: ED, YK; Drafting manuscript: ED, YK; Critical revision of manuscript: ED, YK; Final approval and accountability: ED, YK; Technical or material support: ED, YK; Supervision: ED, YK.

Ethical Approval: Approval for this study was obtained from the Atatürk University Faculty of Medicine Clinical Research Ethics Committee. (Approval number: 2022/6-28)

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