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CLINICAL STUDY

Mitochondrial DNA copy number alterations in familial mediterranean fever patients

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ABSTRACT
OBJECTIVES AND BACKGROUND: Familial Mediterranean Fever (FMF) is characterized by recurrent fever episodes as a result of inflammation of serous membranes. Changes in the number of different mtDNA copy number variations, detected in FMF patients, who developed amyloidosis, might be an important parameter in the understanding of the pathophysiology of the disease.
METHODS: Changes in the mtDNA copy number between 50 patients with FMF, who had M694V homozygote mutation and amyloidosis, and 50 healthy controls, who had not any MEFV mutation or FMF clinical finding, were examined. The 22 MEFV mutations were analyzed by Pyromark Q24 system. Quantitative analysis was performed on RT-PCR. The level of mtDNA was calculated using the delta Ct (ΔCt) of average Ct of mtDNA and nDNA (ΔCt = Ct mtDNA-Ct nDNA) in the same well as an exponent of 2 (2ΔCt).
RESULTS: A significant decrease in the amount of mtDNA was detected in FMF patients with M694V homozygous mutation carriers, who developed amyloidosis compared to the control group ($p < 0.001$).
CONCLUSION: In this study, mitochondrial dysfunction, which has been identified through changes in the mitochondrial genome in many diseases, was identified by showing that the copy number variations of mtDNA in leukocytes also decreased for FMF disease *(Tab. 3, Fig. 1, Ref. 21)*. Text in PDF *www.elis.sk.*
KEY WORDS: familial mediterranean fever, mitochondrial DNA, amyloidosis, MEFV.

Introduction

Familial Mediterranean Fever (FMF) is an autosomal recessive inherited disorder, which is characterized by recurrent fever episodes, abdominal pain, chest pain and arthritis as the result of inflammation of serous membranes (1). In the Middle East and Anatolian populations, where the disease originated, the carrier frequency is 1/6–1/8 and FMF is more common in these regions than in the rest of the world (2). Community of consanguineous marriages also increase the prevalence of FMF in Middle East and Anatolian populations. According to the previous studies, the carrier frequency in Turkish population was 1/5 and the prevalence in Turkish population was 1/1075 (3). Currently, there is a huge number of patients, who are indicated for renal hemodialysis due to chronic renal failure as the result of amyloidosis, because of the delayed diagnosis of FMF and/or disability of the patient's use of colchicine or resistance to colchicine.

In 1997, the International FMF Consortium and the French FMF Consortium reported that mutations in MEFV (MEditerranean FeVer) gene producing marenostrin and pyrine proteins on the 16p13.3 region of chromosome 16 are responsible for the FMF (4, 5). M694V, M680I (G > C), M694I, V726A and E148Q are the most common variants in Anatolia, accounted for 74 % of FMF patients (6, 7). Except for the M694V homozygous mutation, the effect of homozygosity / heterozygosity or compound heterozygosity of other mutations on the MEFV gene in terms of prognosis, has not been clearly determined yet. The mutation most associated with amyloidosis was shown to be the M694V homozygous mutation. Researchers still discuss whether the particularly common E148Q, V726A and R202Q variants are disease-related and should be considered as polymorphism (8–10).

Human mitochondrial DNA (mtDNA) has a double-stranded and circular structure. It contains protein coding and non-coding regions and is composed of about 16,569 nucleotides. There are 37 genes, 2 rRNA, 22 tRNA and 13 mRNA in mtDNA and there is a large number of copies of mtDNA that increase independently in the cell (11). The number of copies can vary from 100 to 100,000 depending on the cell type (12).

Although the pathology of FMF is linked to genetic and biochemical factors, the disease mechanism has not been fully elucidated. In the pathogenesis of some rheumatic diseases, the effect

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Tab. 1. Gender and age characteristics of the patient and control group.

Group	N	Gender	Age
FMF	50	Male(32)/Female(18)	33.4±5.6
Control	50	Male(30)/Female(20)	30.8±4.2

Tab. 2. The sequences of primers of ND-1 and HBB genes.

Gene	Sequence of primer
ND-1/mtDNA (Forward)	TTAGTTGCTTGGTTGTGTATTCC
ND-1/ mtDNA (Reverse)	GAAAAAGGTAAAAAACTCTTTCAAGC
HBB (Forward)	GGAGATGCCTCAGAAACTGC
HBB (Reverse)	AGGTTGGAGGTCGGAAAGTT

of insufficient ATP due to mitochondrial dysfunction was shown in the previous studies (13). The genetic alterations in mtDNA are the most important cause of mitochondrial dysfunction. One of these changes, mtDNA copy number variations, was shown to cause mitochondrial dysfunction in many diseases (14–16). There may also be a pathophysiological basis for the mitochondrial dysfunction with changes in the number of mtDNA copies in FMF.

FMF is a disease with limited biomarkers and amyloidosis that is a frequent and important complication, such as chronic renal failure. For this reason, it is considered that the changes in the number of different mtDNA copy number variations, detected in FMF patients, who developed amyloidosis, are an important parameter in the understanding the pathophysiology of the disease.

Materials and methods

Patient selection

In our study, changes in the mtDNA copy number between 50 patients with FMF, who had M694V homozygote mutation in MEFV gene and amyloidosis, and 50 healthy controls, who did not have any MEFV mutation or FMF clinical finding, were examined. DNA samples, isolated from peripheral blood, were taken from individuals, consulted to Ataturk University Medical Faculty Medical Genetics Department. All of the individuals provided a written informed consent before participating in the study, which was approved by the Ethical Committee of the Ataturk University (decision number; 2016-4/6). The age range of the patient group and the control group was determined to be 18–40. Gender and age characteristics of the patient and control group are summarized in Table 1. Considering the effect of aging on mitochondrial dysfunction, in the selection of the control group, the mean age close to the average age of the patient group was provided. Individuals with autoinflammatory, chronic inflammatory and autoimmune diseases other than FMF were excluded from the study.

Molecular analysis

Blood samples were collected into EDTA tubes. DNA of the patients was extracted by QIAGEN®, EZ1® Advanced XL (Veltek Associates, Inc.) system. The PCR reactions were performed in 50 µl reaction mixture containing 18.5 µl PCR master mix (Qiagen GmbH, Hilden Germany), 2 µl of each primer, 0.5 µl Taq DNA polymerase, 17 µl H_2O and 10 µl mtDNA in a thermal cycler (SensoQuest Labcycler, GmbH, Hilden, Germany) under the following conditions: 95 °C for 15 min (initial denaturation) followed by 32 cycles at 94 °C for 1 min (denaturation), 56 °C for 1 min (annealing), 72 °C for 80 s (extension), and a final extension at 72 °C for 15 min. The 22 MEFV mutations, which are commonly seen in people living in Anatolia were analyzed by QIAGEN®, Pyromark Q24 system with the specific primers. Results were evaluated with PyroMark Q24 Advanced Software, and M694V homozygous patients were selected for the study (Fig. 1). Quantitative analysis was performed on Rotor-Gene-Q (Heidelberg, Germany) RT-PCR to determine the mtDNA copy numbers of the samples. For the analysis, the regions selected from the nuclear DNA (nDNA) and mtDNA were taken as reference. The sequences of the primers, suitable for the NADH dehydrogenase-1 (ND-1) gene identified for the nDNA and the beta-globin

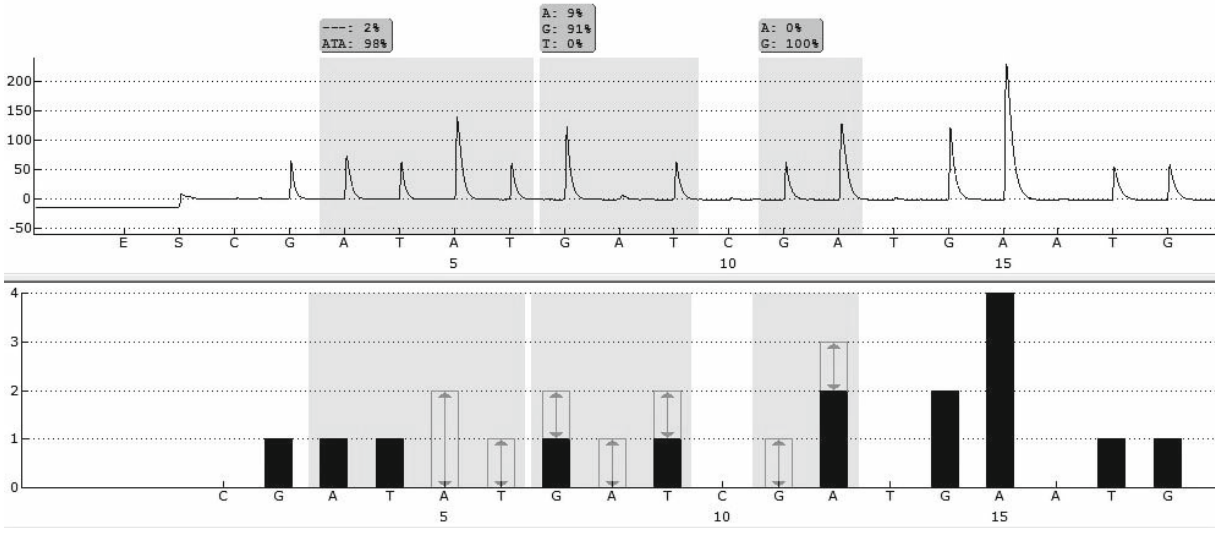

Fig. 1. Pyrosequence image of M694V homozygous FMF patient.

Tab. 3. Statistical analysis of mtDNA copy numbers, detected in FMF patients and control group.

Group	N	Minimum	Maximum	Average	p
FMF	50	3,8637	119.4282	48.190998	< 0.001
Control	50	25,4572	171.2547	80.318280	

(HBB) gene for mtDNA, are shown in Table 2. To determine the quantities of mtDNA and nDNA present in blood samples, the average threshold cycle number (Ct) values of the nDNA and mtDNA were obtained from each case. The level of mtDNA was calculated using the delta Ct (ΔCt) of average Ct of mtDNA and nDNA (ΔCt = Ct mtDNA-Ct nDNA) in the same well as an exponent of 2 ($2^{\Delta Ct}$).

Statistical analysis

The data were evaluated in the SPSS Statistical Package program. Student T test was used to compare two independent groups in terms of numerical variables with normal distribution. Arithmetic mean, standard deviation, median, minimum and maximum values were given as descriptive statistics, $p < 0.05$ was considered statistically significant.

Results

As the result of analyses, the number of mtDNA copies in FMF patients and controls were found to be 48.19 ± 27.3 and 80.31 ± 36.97, respectively. There was also a significant difference in the statistical analysis, between the two groups ($p < 0.001$) (Tab. 3).

Discussion

Mitochondria product almost all the ATP in the cell through oxidative phosphorylation. The most important disadvantage of the oxidative phosphorylation mechanism is that it is a source of reactive oxygen radicals. MtDNA does not have intron regions, histone proteins that provide protection from condensation and external influences, and sufficient DNA repair mechanisms. These conditions make mtDNA susceptible to oxidative damage (17).

Mitochondria are found in every cell type except erythrocytes, and even in the cells of different tissues belonging to the same organism, their number can change. For this reason, our study is based on the ratio of mtDNA to total DNA isolated, rather than the amount of mtDNA per mitochondria.

Previously, many diseases were associated with changes in mtDNA copy number. In relation to aging, it was also found that cognitive functions of women, who detected a high mtDNA in peripheral leukocytes due to increased oxidative stress were better than those that contained lower mtDNA copies in leukocytes (18). Brain tissue is unprotected against oxidative damage, so prolonged oxidative stress may increase dementia development (19). Considering the effect of age on the number of mtDNA copies, attention has been paid to the fact that the age and the average age of the patients and the control groups examined in our study are close to each other.

Mitochondrial dysfunction has also been associated with autism spectrum disorders (OSB), which are common behavioral disorders (19, 20). Unlike our study, there was an increase in mtDNA copy numbers in the studies related to OSB. This is explained by an increase in replication and a decrease in degradation as the result of the oxidative stress response (14). In the study about Huntington's disease, the number of mtDNA copies increased in patients, who had not yet started the disease clinically, but had a family history and had an increased CAG repeat in themselves; a decrease in the number of mtDNA copies in patients, who are in the onset of the clinical progression; whereas the number of mtDNA copies decreased in the advanced stages of the disease (21). In our study, patients were not classified according to the disease phase. Investigations of mtDNA copy numbers in patients with family history and M694V homozygote mutations, but do not have attacks yet; and the patients, who had attacks and clinical signs are thought to be able to provide insight into the changes in mtDNA copy number that can be seen in the life span of FMF patients.

MtDNA alterations have been investigated in some rheumatologic diseases, although there has been no previous study of mtDNA copy number or other changes in mtDNA in relation to FMF. In this context, the study with patients, who developed lupus nephritis followed by SLE showed a significant decrease in the number of mtDNA copies as in our study (13).

As conclusion, mtDNA copy number has the potential to be used as biomarker for many diseases and conditions. This study showed that FMF disease was inversely proportional to the number of mtDNA copies. In FMF patients, extended studies with different patient groups in relation to mtDNA copy number change are also important in order to clarify the molecular mechanism of the disease and to fully understand the effect of mitochondrial dysfunction on FMF.

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