Mitochondrial dynamics and biogenesis indicators may serve as potential biomarkers for diagnosis of myasthenia gravis

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Abstract. Due to challenges in diagnosing myasthenia gravis (MG), identifying novel diagnostic biomarkers for this disease is essential. Mitochondria are key organelles that regulate multiple physiological functions, such as energy production, cell proliferation and cell death. In the present study, Mfn1/2, Opa1, Drp1, Fis1, AMPK, PGC-1α, NRF-1 and TFAM were compared between patients with MG and healthy subjects to identify potential diagnostic biomarkers for MG. Blood samples were collected from 50 patients with MG and 50 healthy subjects. The participants' demographic information and routine blood test results were recorded. Mitochondrial dynamics were evaluated and levels of Mfn1/2, Opa1, Drp1, Fis1, AMPK, PGC-1α, NRF-1 and TFAM were determined in peripheral blood mononuclear cells using western blotting and reverse transcription-quantitative PCR, respectively. Receiver operating characteristic curve analysis was used to evaluate the diagnostic accuracy of these indicators. The areas under the curve values of Mfn1/2, Opa1, Drp1, Fis1,AMPK, PGC-1α, NRF-1 and TFAM were 0.5408-0.8696. Compared with control subjects, mRNA expression levels of Mfn1/2, Opa1, AMPK, PGC-1α, NRF-1 and TFAM were lower, while those of Drp1 and Fis1 were higher in patients with MG. The protein expression levels of all these molecules were lower in patients with MG than in control subjects. These results suggested that mitochondrial dynamics and biogenesis indicators may be diagnostic biomarkers for MG.

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Abbreviations: Ab, antibody; AMPK, AMP-activated protein kinase; Drp, dynamin-related protein; Fis, fission; Mfn, mitofusion; MG, myasthenia gravis; mtDNA, mitochondrial DNA; NRF-1, nuclear respiratory factor-1; OMM, outer mitochondrial membrane; Opa 1, optic atrophy type 1; PGC-1α, peroxisome proliferator-activated receptor-γ co-activator-1α; ROC, receiver operating characteristic; TFAM, mitochondrial transcription factor A

Key words: myasthenia gravis, mitochondrial dynamics, biogenesis, peripheral blood mononuclear cells, biomarkers

Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by dysfunctional transmission of nerve impulses to muscles (1), which impedes eyelid movement, facial expression, chewing, talking, swallowing and breathing. The global incidence of MG is 1.7-21.3/1,000,000 individuals per year (2). Although studies have shown that the mortality rate of MG is currently lower than two decades ago (3,4), MG still impacts the quality of life of patients. In current clinical practice, MG diagnosis is dependent on disease stage and involves clinical examination based on serum autoantibody detection. The specific diagnostic tools evaluate levels of anti-acetylcholine receptor (AChR) and anti-muscle-specific tyrosine kinase (MuSK) Abs (5). However, ~10% of patients with MG test negative for both anti-AChR and anti-MuSK Abs (6). Therefore, developing novel methods to diagnose MG is vital.

Mitochondria are abundant in muscle cells and provide most of the energy required to maintain daily activity of the human body (7). They are key for energy production and cell proliferation and death (8). Mitochondrial function is regulated and depends on structure, dynamics and biogenesis (9). Mitochondria serve a key role in improving the metabolic quality and plasticity of skeletal muscles by maintaining biogenesis, dynamics and autophagy/mitophagy (10).

Mitofusion 1 (Mfn1), Mfn2, optic atrophy type 1 (Opa1), dynamin-related protein 1 (Drp1) and fission 1 (Fis1) are key factors associated with mitochondrial fusion and fission (11).

Mfn1 and Mfn2 are located in the outer mitochondrial membrane (OMM) and regulate mitochondrial fusion. Opa1 is a member of the dynamin family of mechanoenzymes that are localized in the inner MM, where they regulate mitochondrial fusion (12).

Previous studies have indicated that regulation of Mfn2 to restore mitochondrial homeostasis inhibits development of diabetic cardiomyopathy (13,14). Drp1 dissociates in the cytosol, while Fis1 is anchored to the OMM. Specific signals induce transfer of Drp1 from the cytosol to the surface of the organelle where it interacts with Fis1 to complete organelle division (12,15). This physiological process is known as mitochondrial dynamics (8).

AMP-activated protein kinase (AMPK) is an energy metabolism receptor that serves an important role in maintaining balanced energy metabolism in cells (16). Peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) is the primary transcriptional regulator of mitochondrial biogenesis, respiration and oxidative phosphorylation (17). Nuclear respiratory factor-1 (NRF-1) is a nuclear transcription factor that stimulates expression of nuclear genes to enhance mitochondrial respiratory function. Mitochondrial transcription factor A (TFAM), a DNA-binding protein, controls mitochondrial metabolism and dysfunction by regulating the transcription of its genome and organizing mitochondrial DNA (mtDNA) (18). PGC-1α activates TFAM by serving as a co-transcription factor of NRF-1, thereby regulating mitochondrial biogenesis (19).

Previous studies have shown that mitochondria serve a role in the development of rare neuromuscular diseases, such as Duchenne and Becker muscular dystrophy (20,21). Mitochondrial dysfunction affects muscle function, leading to atrophy (22). Muscle weakness is the primary symptom of MG. Both muscle contraction and relaxation require mitochondria for energy supply; the dynamic balance and biogenesis of mitochondria are key for these processes (23). Therefore, it was hypothesized that mitochondrial dynamics and biogenesis factors may serve as diagnostic biomarkers for MG. The aim of the present study was to investigate the differences in mitochondrial dynamics and biogenesis between patients with MG and healthy individuals. Receiver operating characteristics (ROC) curves were plotted using reverse transcription-quantitative (RT-q)PCR data to evaluate the diagnostic value of mitochondria-associated genes, as well as to determine whether mitochondrial dynamics and biogenesis factors can serve as diagnostic biomarkers for MG.

Materials and methods

Participants. Patients with MG (19 males and 31 females) were enrolled from The First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China) between August 2018 and February 2019. Samples from 50 healthy volunteers (25 males, 25 females) were also included in the present cross-sectional study. The following inclusion criteria were used: i) Age, 14-75 years, ii) willing to participate in the study and iii) examination by personnel trained in diagnosis of MG class IIb (based on the clinical classification proposed by the MG Foundation of America) (24). The following exclusion criteria were used: i) Hormone, immunosuppression, plasmapheresis, or intravenous γ globulin treatment during the previous 3 months, ii) serious infectious disease or subsequent complications (such as mental illness; cerebrovascular, heart or liver disease; kidney failure; or malignant tumor) and iii) participation in another clinical study in the past 3 months. All participants understood the experimental procedure and provided written informed consent. Written informed consent was obtained from the parents/guardians of all participants <18 years old. All procedures involving human subjects were approved by the Academic Ethics Committee of The First Affiliated Hospital of Guangzhou University of Chinese Medicine.

Isolation of human peripheral blood mononuclear cells (PBMCs). Peripheral blood samples (5 ml) were collected in an anticoagulant tube. Within 1 h of sample collection, PBMCs were isolated using Ficoll-Paque™ gradient (GE Healthcare). An equal volume of phosphate buffer was added to dilute the blood sample, which was added to the Ficoll-Paque separating solution and centrifuged at 600 x g for 20 min at 25˚C. The samples were separated into four layers. PBMCs were collected from the second layer (mononuclear cells). PBMCs were washed three times with phosphate-buffered saline and centrifuged (600 x g, 10 min, 4˚C), and then stored at -80˚C (Fig. 1).

Total RNA extraction. Total RNA was isolated from PBMCs using TRIzol® reagent, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). TRIzol reagent (1 ml) and chloroform (0.2 ml) were added to each sample tube. The tubes were mixed for 15 sec, allowed to stand for 3 min and centrifuged at 600 x g for 15 min at 4˚C. The upper aqueous phase was carefully aspirated and transferred to a new tube. An equal volume of isopropanol was added and samples were mixed at 25˚C for 20 min. The samples were centrifuged at 13,000 x g for 10 min at 4˚C to precipitate the RNA and the supernatant was removed. The sediment was washed with 1 ml pre-cooled 75% ethanol and centrifuged again (600 x g, 5 min, 4˚C). After discarding the supernatant, the RNA pellet was dissolved in 30 μl diethyl pyrocarbonate-treated water and stored at -80˚C.

RT-qPCR evaluation of target mRNA expression in PBMCs. Total RNA was extracted from PBMCs using TRIzol® reagent as aforementioned and reverse-transcribed to cDNA using PrimeScript™ RT Master Mix (cat. no. RR036Q; Takara Bio, Inc.) according to the manufacturer's instructions. The mRNA expression levels were measured using SYBR Green Master Mix (cat. no. RR036A; Takara Bio, Inc.) on a CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions, using the following thermocycling conditions: Initial denaturation at 95˚C for 20 sec, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec and 70˚C for 1 sec. The target gene sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and primer sequences for all target genes are listed in Table I. Relative expression was determined using the $2^{-\Delta\Delta Cq}$ method (25). GAPDH was used as the reference gene.

Western blotting evaluation of protein expression in PBMCs. PBMCs were lysed on ice with RIPA lysis buffer

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'
Mfn1	ATGTAACGGACGCCAATC	ATCTTTAGCTTCTACTCCCACT
Mfn2	TGCAGGTGTAAGGGACGATT	GAGGCTCTGCAAATGGGATG
Opa1	TGTCCTCCGCAAAGTCAT	TGCTTGGGAGACCCTACA
Drp1	CAAAGCAGTTTGCCTGTGGA	TCTTGGAGGACTATGGCAGC
Fis1	CCAGGTAGAAGACGTAATCCC	GTCCAAGAGCACGCAGTTT
AMPK	TTGAAACCTGAAAATGTCCTGCT	GGTGAGCCACAACTTGTTCTT
PGC-1α	TCAGTCCTCACTGGTGGACA	TGCTTCGTCGTCAAAAACAG
NRF-1	GGTGCAGCACCTTTGGAGAA	CCAGAGCAGACTCCAGGTCTTC
TFAM	CACATTTTCCACCTGGTGAT	CACTCCGCCCTATAAGCATC
GAPDH	AAGAAGGTGGTGAAGCAGG	GTCAAAGGTGGAGGAGTGG

Mfn, mitofusion; Opa, optic atrophy; Drp, dynamin-related protein; Fis, fission; AMPK, AMP-activated protein kinase; PGC, peroxisome proliferator-activated receptor-γ co-activator; NRF, nuclear respiratory factor; TFAM, mitochondrial transcription factor A.

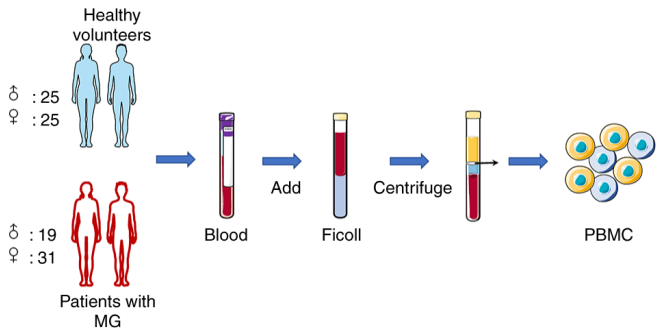

Figure 1. Isolation of human PBMCs. PBMC, peripheral blood mononuclear cell; MG, myasthenia gravis.

(cat. no. 78510; Thermo Fisher Scientific, Inc.) and centrifuged at 13,000 x g for 15 min at 4˚C, and then supernatant was collected. Protein concentration was determined using the bicinchoninic acid method. An equal amount (30 μg, 20 μl) of protein was loaded/lane and samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples were transferred onto polyvinylidene difluoride membranes using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc.) and blocked at 25˚C with 5% non-fat powdered milk for 1.5 h. The membranes were incubated overnight at 4˚C with the following primary antibodies (all Abcam): Anti-Mfn1 (cat. no. ab129154; rabbit; 1:2,000 in 5% non-fat dry milk), anti-Mfn2 (cat. no. ab124773; rabbit; 1:1,000 in 5% non-fat dry milk), anti-Opa1 (cat. no. ab42364; rabbit; 1:2,000 in 5% BSA), anti-Drp1 (cat. no. ab219596; rabbit; 1:2,000 in 5% BSA), anti-Fis1 (cat. no. ab229969; rabbit; 1:200 in 5% non-fat dry milk), anti-AMPK (cat. no. ab80039; mouse; 1:1,000 in 5% non-fat dry milk), anti-phosphorylated-AMPK (cat. no. ab133448; rabbit; 1:1,000 in 5% non-fat dry milk), anti-PGC-1α (cat. no. ab54481; rabbit; 1:1,000 in 5% non-fat dry milk), anti-NRF-1 (cat. no. ab34682; rabbit; 1:1,000 in 5% non-fat dry milk), anti-TFAM (cat. no. ab176558; rabbit; 1:1,000 in 5% non-fat dry milk) and anti-GAPDH (cat. no. ab8245; mouse; 1:5,000 in 5% non-fat dry milk). The membranes were then incubated with goat anti-rabbit (cat. no. ab7085; 1:3,000 in 5% non-fat dry milk) or goat anti-mouse (cat. no. ab7063; 1:3,000 in 5% non-fat dry milk) secondary antibodies for 90 min at 25˚C, then washed three times for 5 min each using Tris-buffered saline and polysorbate 20 (Beijing Solarbio Science & Technology Co., Ltd.). Protein bands were visualized using Clarity™ Western ECL Substrate kit (Bio-Rad Laboratories, Inc.). The chemiluminescent signal was captured using a ChemiDoc™ XRS+ system and resulting bands were analyzed using Image Lab software version 3.0 (both Bio-Rad Laboratories, Inc.).

Statistical analysis. The sensitivity and specificity of variables for MG diagnosis were determined by ROC curve analysis using a non-parametric approach. The optimal cutoff values were selected based on those that minimized the sensitivity-specificity difference and maximized the discriminating power of the tests. All data were repeated three times and analyzed using SPSS 22.0 (IBM Corp.) and are expressed as the mean ± standard deviation. Unpaired t-test was used to compare differences between control and MG. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Participant information. A total of 50 healthy volunteers (25 males and 25 females; mean age, 38.92±14.76 years) were recruited for the control group and 50 patients with MG (19 males and 31 females; mean age, 42.58±13.76 years) were recruited for the MG group. The clinical characteristics of participants are listed in Table II (raw data are shown in Table SI). A higher number of women than men were affected by MG, which was consistent with clinical reports (26,27), and 66% of patients with MG were aged 30-60 years. According to Table II, the proportion of MG patients with a disease span of 0-6 years was 88%. The patients exhibited dysphagia, chewing weakness, thymectomy and thyroid dysfunction (Table II). The hematology results of the patients with MG are shown in Table III. Routine blood examination values were all within normal ranges. The results provide comprehensive information on the participants.

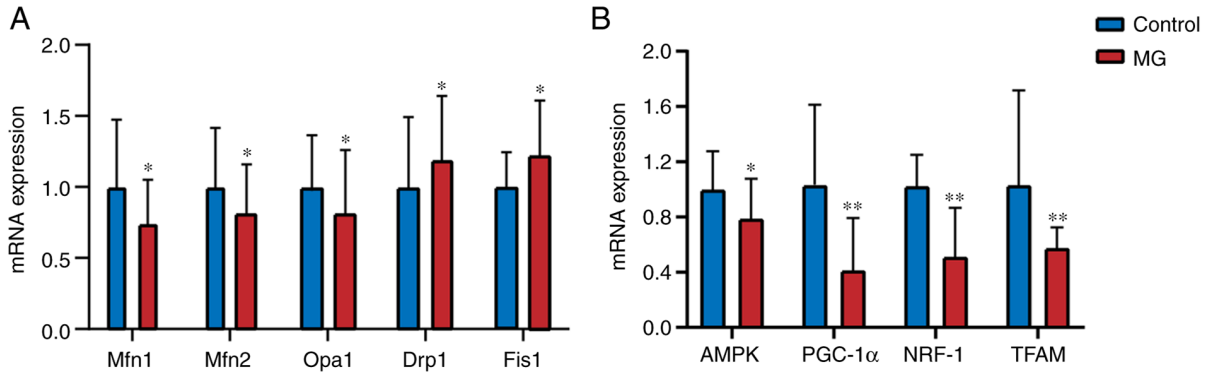

Figure 2. mRNA expression levels of mitochondrial fusion/fission-associated genes in peripheral blood mononuclear cells. (A) Mfn1/2, Opa 1, Fis 1, Drp 1, (B) AMPK, PGC-1α, NRF-1 and TFAM, mRNA expression levels in control and MG group. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control. Mfn, mitofusion; Opa, optic atrophy; Drp, dynamin-related protein; Fis, fission; AMPK, AMP-activated protein kinase; PGC, peroxisome proliferator-activated receptor-γ co-activator; NRF, nuclear respiratory factor; TFAM, mitochondrial transcription factor A.

Table II. Clinical characteristics of patients and controls.

Characteristic	MG (n=50)	Control (n=50)
Sex		
Male	19	25
Female	31	25
Age, years		
14-29	9	23
30-44	20	6
45-59	16	17
≥60	5	4
Course of disease, months		
0-36	36	-
37-72	8	-
73-108	4	-
>108	2	-
Symptom (+)		-
Neostigmine	50	-
AchR-Ab	44	-
MuSK-Ab	30	
Dysphagia	25	-
Chewing weakness	11	-
Thymectomy	28	-
Thyroid dysfunction	10	-

MG, myasthenia gravis; AchR, acetylcholine receptor; Ab, antibody; MuSK, muscle-specific tyrosine kinase.

mRNA expression of Mfn1, Mfn2, Opa1, Drp1, Fis1, AMPK, PGC-1α, NRF-1 and TFAM measured by RT-qPCR. mRNA expression levels of Mfn1, Mfn2 and Opa1 were significantly lower in the MG than the control group (P<0.05; Fig. 2; Table SII). By contrast, mRNA expression of Drp1 and Fis1 was higher in the MG group than the control (P<0.05). In addition, mRNA expression of AMPK, PGC-1α, NRF-1 and TFAM was significantly lower in the MG group than in the control (P<0.05). The results of 6 MG patients who were anti-AChR-negative are shown in Table SIII. According to the comparison of the results, there were differences in mRNA expression between control group and MG group patients (including antibody-negative patients).

Diagnostic value of Mfn1, Mfn2, Opa1, Drp1, Fis1, AMPK, PGC-1α, NRF-1 and TFAM. To determine whether mitochondria-associated genes serve as diagnostic biomarkers for MG, ROC curves were plotted using RT-qPCR data (Fig. 3). In ROC curve analysis (28), the area under the curve (AUC) quantifies the diagnostic potential of each candidate biomarker with a high AUC indicating a more accurate distinction between patients with MG and controls. AUCs were 0.8628 for PGC-1α, 0.8696 for NRF-1, 0.8072 for TFAM, 0.6488 for Mfn1, 0.6441 for Mfn2, 0.6212 for Opa1, 0.6468 for Drp1, 0.6604 for Fis1 and 0.5408 for AMPK (Fig. 3A-I). The results showed that mitochondrial energy metabolism (as measured by PCG-1α, NRF-1 and TFAM) had higher diagnostic value for MG.

Evaluation of Mfn1, Mfn2, Opa1, Drp1, Fis1, AMPK, PGC-1α, NRF-1 and TFAM expression by western blotting. Western blotting was performed to determine whether proteins were differentially expressed between control and MG groups. Mitochondrial dynamics-associated proteins (Mfn1, Mfn2, Opa1, Drp1 and Fis1) were expressed at significantly lower levels in PBMCs from patients with MG than in control subjects (P<0.05; Fig. 4). Mitochondrial biogenesis-associated proteins (AMPK, PGC-1α, NRF-1 and TFAM) were also expressed at lower levels in patients with MG than in control subjects (P<0.05; Fig. 5). The results indicate that these mitochondrial markers may have diagnostic value for MG.

Discussion

Mitochondria are abundantly present in skeletal muscle fibrils, which require large amounts of ATP for contraction and diastolic movement (29). Mitochondria are semi-autonomous organelles that are key sites of tricarboxylic acid cycle reactions; their normal function determines whether skeletal muscles contract freely and flexibly (30). The accepted mechanism of skeletal muscle contraction (sliding filament theory) asserts that, when stimulated by neurotransmitters and in the presence

Table III. Hematology results of patients with myasthenia gravis (mean ± SD, n=50).

Variable	Male (n=19)	Normal male range	Female (n=31)	Normal female range
WBC, $10^3/\mu l$	8.59±4.63	4.0-10.0	9.94±3.13	4.0-10.0
RBC, $10^6/\mu l$	4.45±0.57	4.0-5.5	4.58±0.62	3.5-5.0
PLT, $10^3/\mu l$	227.11±60.22	100.0-300.0	229.78±41.86	100.0-300.0
Hb, g/l	130.89±19.18	120.0-160.0	130.42±18.06	110.0-150.0

WBC, white blood cell; RBC, red blood cell; PLT, platelet; Hb, hemoglobin.

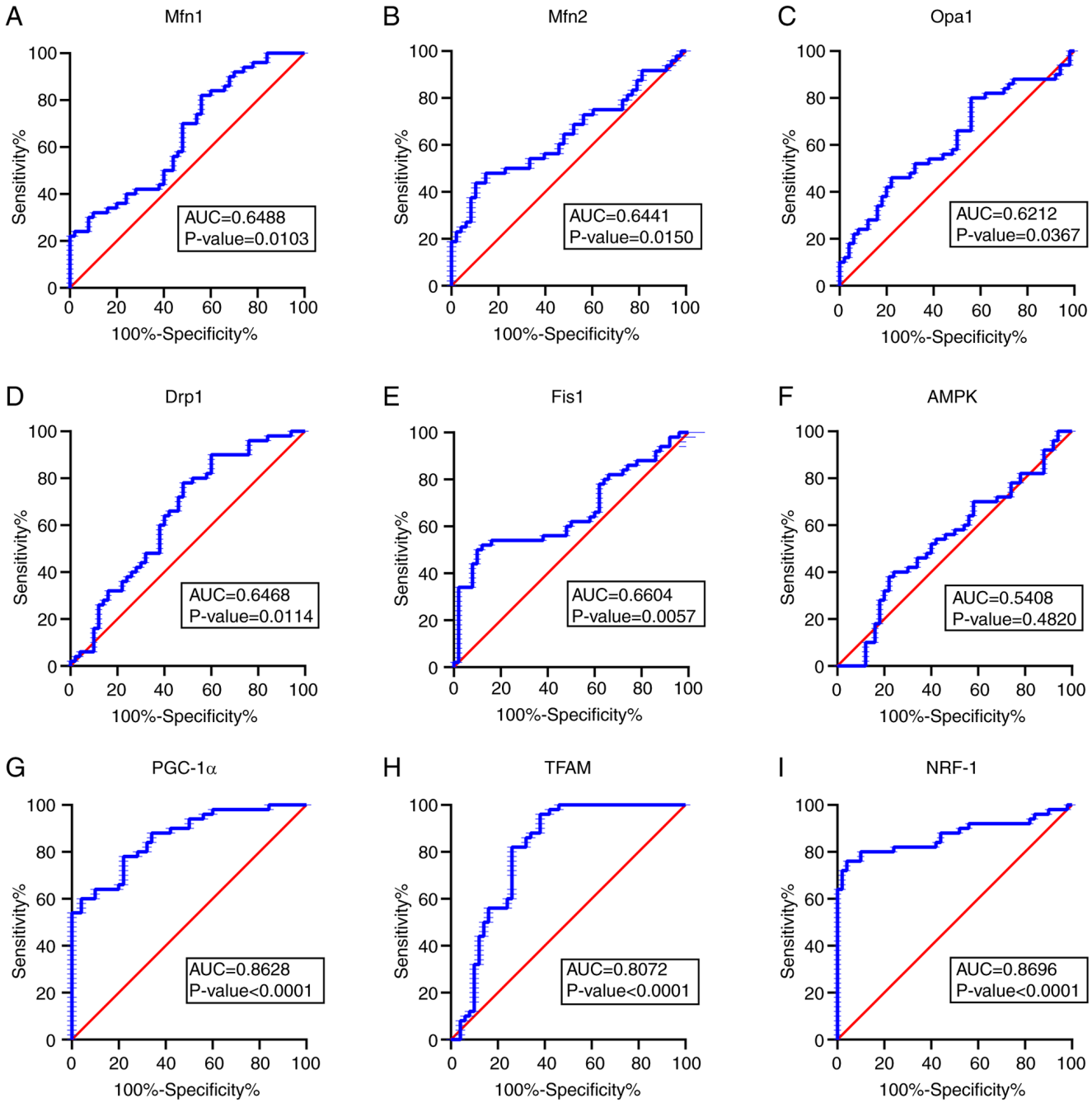

Figure 3. Receiver operating characteristic curves for mitochondrial dynamics- and biogenesis-associated mRNAs. AUC of (A) Mfn 1 is 0.6488, of (B) Mfn 2 is 0.6441, of (C) Opa 1 is 0.6212, of (D) Drp 1 is 0.6468, of (E) Fis 1 is 0.6604, of (F) AMPK is 0.5408, of (G) PGC-1α is 0.8628, of (H) TFAM is 0.8072 and of (I) NRF-1 is 0.8696. Analysis based on the reverse transcription-quantitative PCR results in mRNAs plotted as sensitivity versus specificity. AUC>0.5 was considered significant. AUC, area under the curve; Mfn, mitofusion; Opa, optic atrophy; Drp, dynamin-related protein; Fis, fission; AMPK, AMP-activated protein kinase; PGC, peroxisome proliferator-activated receptor-γ co-activator; NRF, nuclear respiratory factor; TFAM, mitochondrial transcription factor A.

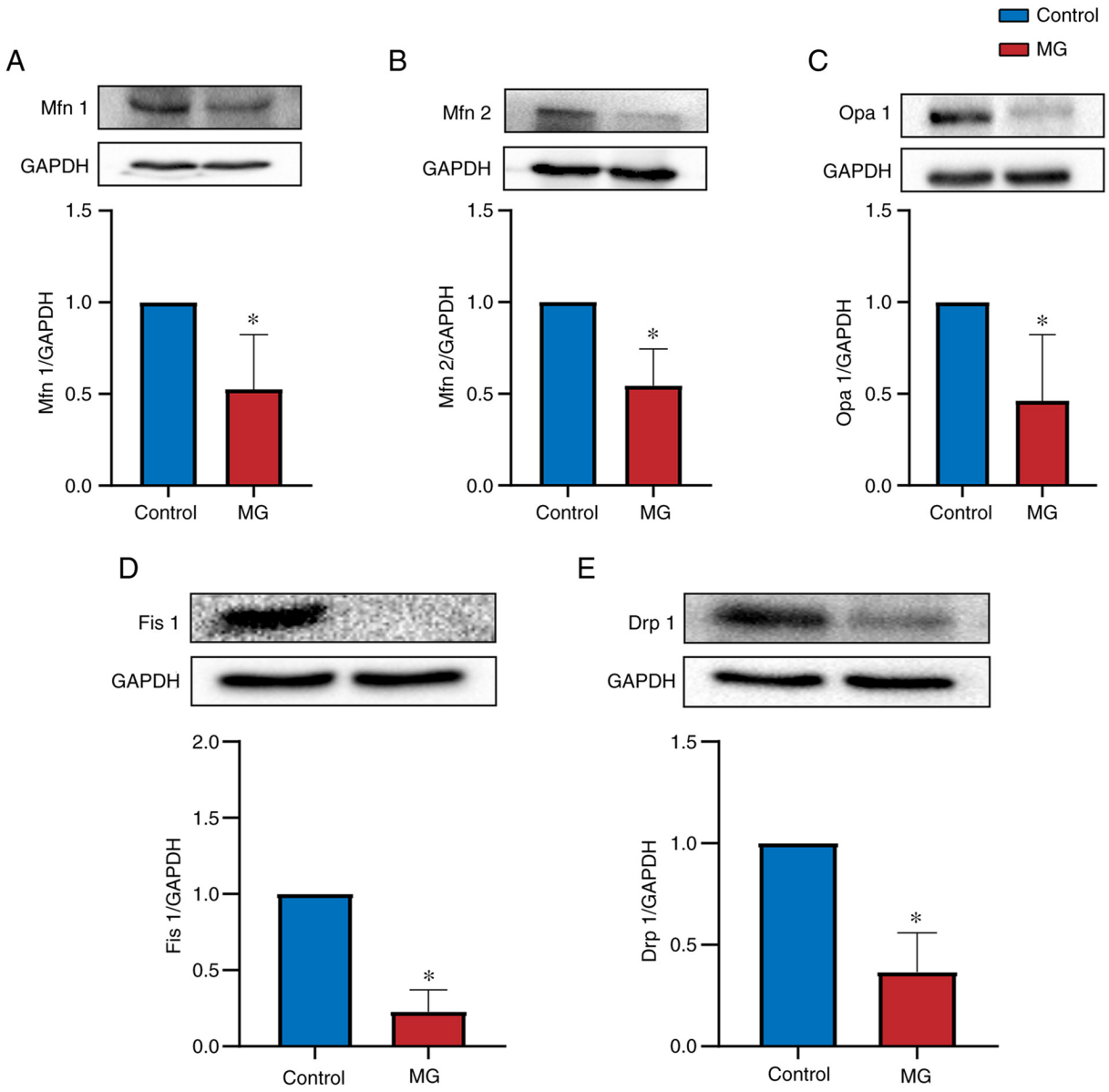

Figure 4. Levels of mitochondrial fusion/fission-associated protein in peripheral blood mononuclear cells. (A) Mfn1, (B) Mfn2, (C) Opa 1, (D) Fis 1 and (E) Drp 1 protein expression in control and MG group. Data are presented as the mean ± SD. *P<0.05 vs. control. Mfn, mitofusion; Opa, optic atrophy; Drp, dynamin-related protein; Fis, fission; MG, myasthenia gravis.

of ATP hydrolysis, muscle myosin and actin perform muscle contraction by sliding on muscle fibers, leading to overall macro fiber shortening and densifying (31). Furthermore, studies (32,33) have shown that muscle cells in patients with MG are highly sensitive to energy deficiency, which affects signal transduction and normal physiological activity of the neuromuscular junction. Histopathological analysis of extra-ocular muscle tissues of patients with MG has shown that myopathic features predominantly include substitution of muscle fibers by adipocytes and mitochondrial dysfunction at the ultrastructural level (34). Another study reported mitochondrial dysfunction in a patient with early-stage muscular dystrophy; these abnormal mitochondria were susceptible to further damage following sarcolemma injury (35). These results suggest that mitochondrial and neuromuscular disease are associated. Insufficient mitochondrial ATP synthesis leads to development of muscle movement disorders and clinical MG symptoms, such as limb weakness, eyelid ptosis, chewing weakness, dysphagia and respiratory muscle paralysis (Fig. 6).

The most common method for MG diagnosis in clinical practice includes assessment of symptoms and signs of MG and a positive test for specific autoantibodies (36). However, a portion of patients with MG are not diagnosed as anti-AChR or anti-MuSK positive; thus, this method should be complemented.

By contrast with previous studies (37,38) on MG that have concentrated on the immune system, the present study investigated mitochondrial dysfunction in muscles as a potential mechanism for MG. A large number of patients were needed to achieve reasonable results but the invasive nature of muscle tissue sampling causes pain and imposes a psychological burden on participants, resulting in reluctance to enroll in this type of study. This was a limitation of the present study. However, collecting blood samples for PBMC

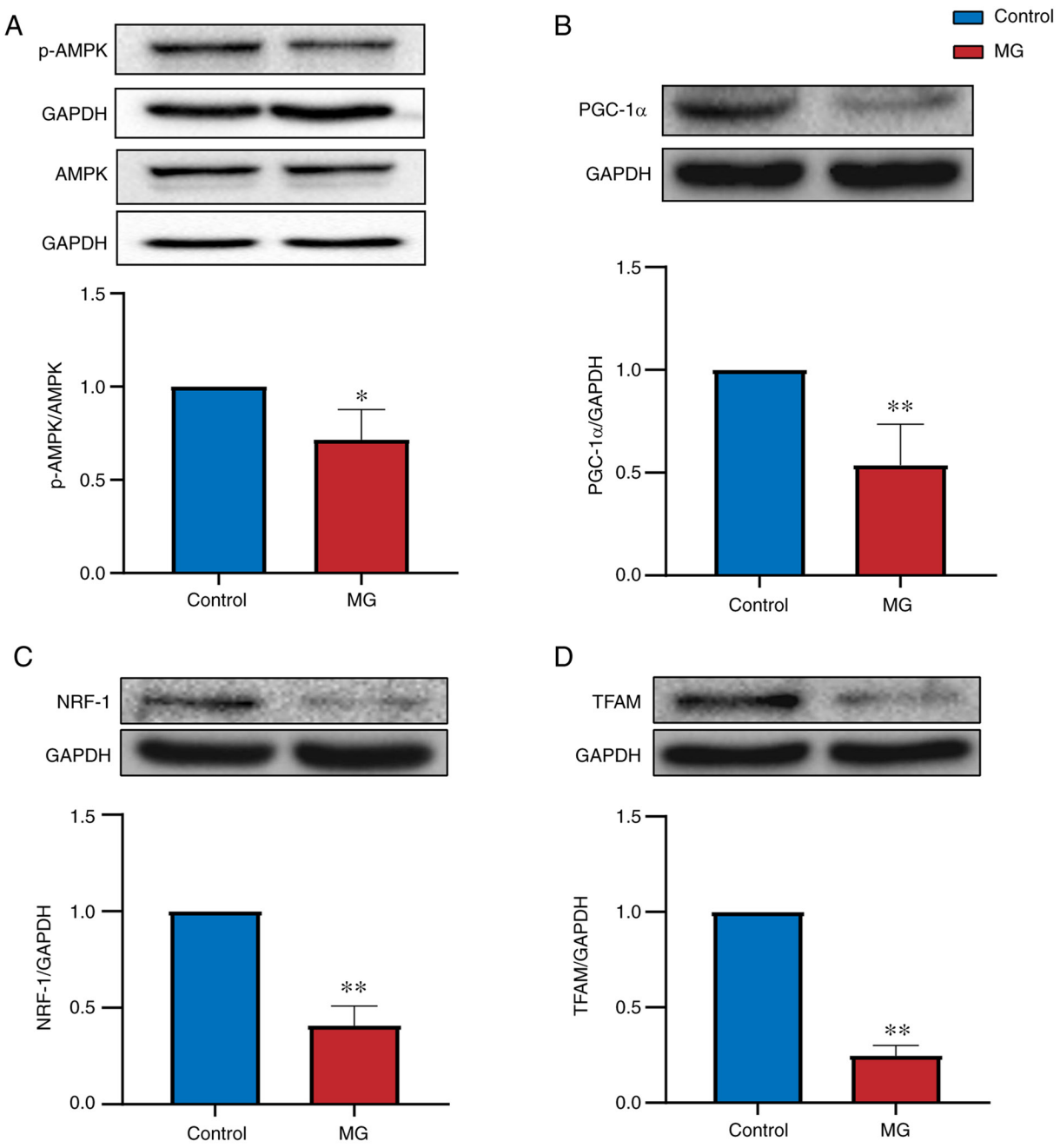

Figure 5. Expression levels of mitochondrial biosynthesis-associated protein in peripheral blood mononuclear cells. (A) AMPK, (B) PGC-1α, (C) NRF-1 and (D) TFAM protein expression levels in control and MG group. Data are presented as the mean ± SD. *P<0.05, **P<0.01 vs. control. AMPK, AMP-activated protein kinase; PGC, peroxisome proliferator-activated receptor-γ co-activator; NRF, nuclear respiratory factor; TFAM, mitochondrial transcription factor A; p-, phosphorylated; MG, myasthenia gravis.

isolation is relatively painless, involves a simple procedure and has high patient compliance, which increases the feasibility of long-term research. PBMCs are used to identify cellular dysfunction associated with the pathophysiology of Parkinson's disease, a neurodegenerative disorder, such as decreased proteasome activity and mitochondrial dysfunction (39). Yalçınkaya *et al* (40) reported that gene expression analysis using PBMCs is a simple diagnostic method for Parkinson's disease. This research provided a reference for the present study to analyze the diagnostic value of MG by PBMCs.

Mitochondrial fission and fusion are key for immature cell proliferation as they provide cells with an adequate number of mature mitochondria for effective bioenergy genesis (41). Mitochondrial fusion helps mitochondria resist oxidative stress-induced damage (42). Studies have shown that mitochondrial fusion and fission impairment may affect mitochondrial function and lead to cardiomyocyte death (43,44).

In the present study, mRNA and protein levels of fusion-associated genes Mfn1/2 and Opa1 were decreased in patients with MG compared with control subjects. Mfn1 promotes fusion of tethering-adjacent mitochondria in coordination with Opa1, whereas Mfn2 acts independently. Mitochondria of cardiomyocytes in Mfn-2-deficient mice are pleiomorphic, enlarged and exhibit functional deterioration (15,45).

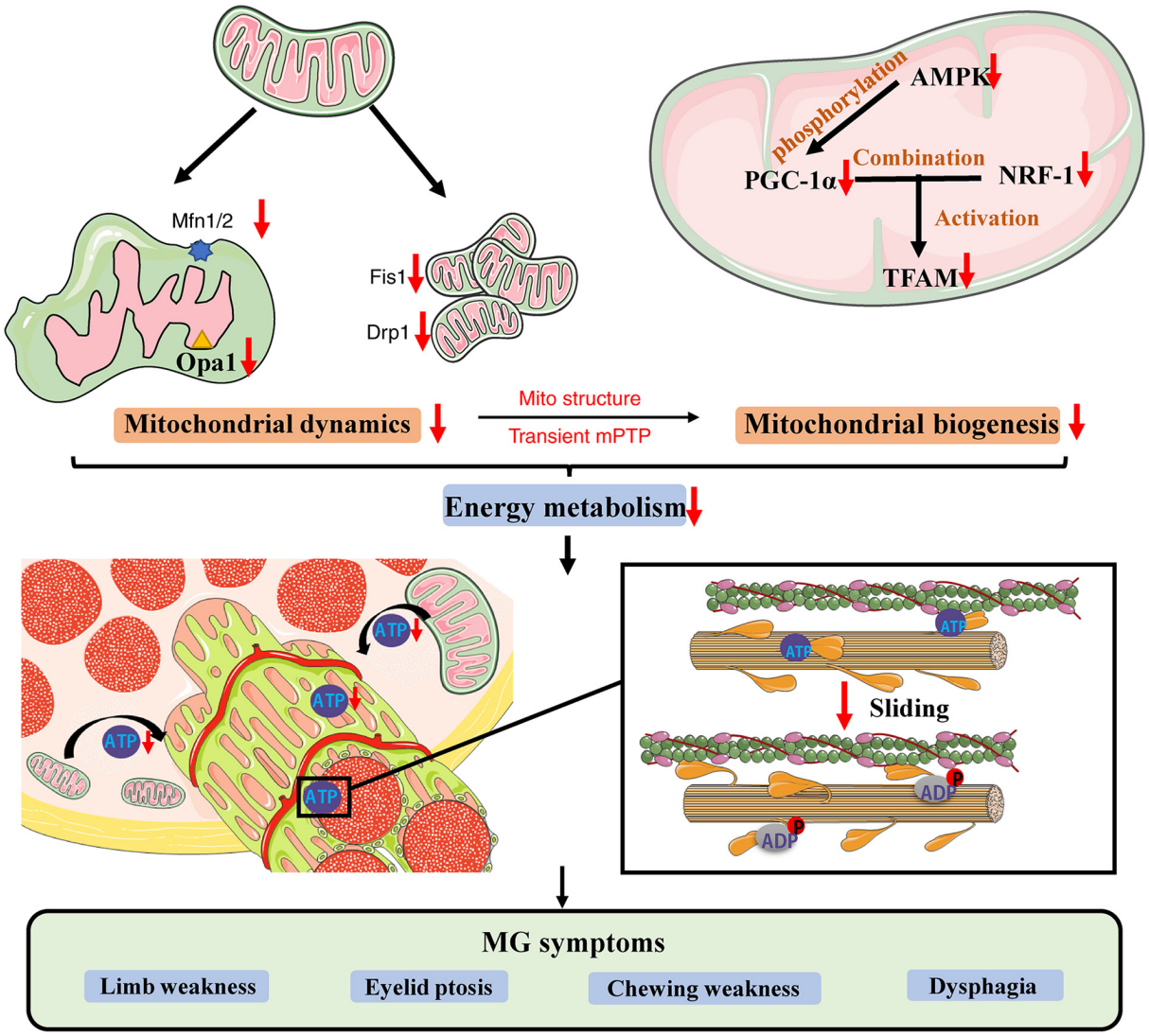

Figure 6. Schematic diagram of mitochondrial dynamics and biogenesis associated with MG. Decreased Mfn1/2, Opa1, Fis1 and Drp1 proteins leads to imbalance of mitochondrial fusion and fission, affecting mitochondrial structure and regulation of mitochondrial permeability transition pore channels. AMPK modulates activation of PGC-1α and binding to NRF-1 to activate TFAM. Decreased expression of these proteins decreases mitochondrial biosynthesis, energy generation and ATP synthesis, affecting the binding of myosin and actin and causing MG symptoms, such as limb weakness, eyelid ptosis, chewing weakness and dysphagia. MG, myasthenia gravis; Mfn1/2, mitofusion1/2; Opa1, optic atrophy type 1; Drp1, dynamin-related protein 1; Fis1, fission 1; AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferators activated receptor γ coactivator 1α; NRF-1, nuclear respiratory factor-1; TFAM, mitochondrial transcription factor A; mPTP, mitochondrial permeability transition pore.

Santel *et al* (46) found that the highest mRNA levels of Mfn1 and Mfn2 are present in energy-demanding tissue, such as skeletal muscle, heart and brain, which demonstrates the role of mitochondrial fusion in the energy supply chain.

Fission-associated gene products include Fis1 and Drp1. Drp1 translocates to the OMM following signaling from cytosolic GTPase and active fission sites (45). Fis1 recruits Drp1 to the mitochondria to regulate fission, which is associated with skeletal muscle mass. The impairment of mitochondrial fission has been shown to result in muscle atrophy (8,47). These two proteins were expressed at lower levels in patients with MG than in control subjects. However, in the present study, mRNA expression levels of Drp1 and Fis1 were higher than expected compared with protein levels. Fis1 was barely detected in patients with MG, as confirmed by repeat testing. Therefore, it was hypothesized that other factors may have influenced the translation process to decrease protein synthesis. Untranslated regions (UTRs) determine the fate of proteins by regulating their interactions. In most cases, single-stranded miRNAs are not fully complementary to the 3'-UTR of their target mRNA, thus blocking translation and regulating gene expression. Synthetic 5'-UTR RNA structures regulate protein translation in mammalian cells (48,49). Circular RNAs also exhibit a potent translation regulatory function via their sponge function (50). These mechanisms only affect protein levels, not mRNA stability. Further investigation is required to determine the mechanism underlying differences in mRNA and protein expression levels.

Mitochondrial dynamics and biogenesis are reciprocally coupled. Mitochondrial fission-associated proteins induce opening of the mitochondrial permeability transition pore channel, which leads to changes in mitochondrial membrane potential and reactions in the mitochondrial respiratory chain (51), which enhance mitochondrial biogenesis.

Mitochondrial biogenesis serves a vital role in metabolic health and plasticity. AMPK is an energy metabolism receptor that phosphorylates PGC-1α and activates SIRT1 by increasing cellular NAD^+ levels. Furthermore, AMPK leads to increased expression of PGC-1α (17). SIRT1 has been shown to interact with PGC-1α to enhance mitochondrial biogenesis (52). The overexpression of PGC-1α is an effective therapy for age-associated muscle loss. In addition, PGC-1α-deficient mice exhibit neurodegeneration, suggesting that PGC-1α may be involved in the pathogenesis of neuromuscular disease (53,54). PGC-1α interacts with NRF-1, a member of the NRF-1 Cap'n'collar-Basic leucine zipper protein family of nuclear transcription factors, to increase TFAM expression and regulate mitochondrial biosynthesis. NRF-1 stimulates nuclear gene expression to promote mitochondrial respiratory reactions. TFAM is a member of the high-mobility-group-box domain-containing protein family that initiates transcription of mtDNA. Conditional knockout of TFAM in dopaminergic neurons in MitoPark mice results in decreased mtDNA levels (55,56). Therefore, biosynthesis promotes mitochondrial ATP synthesis and increases the number of mitochondria to provide an energy reservoir for skeletal muscle contraction (57,58).

Preliminary work by our group on the gastrocnemius muscle tissue of a rat model of autoimmune MG, in which mitochondria are vacuolated, showed that the cristae were broken, expression levels of fusion- and fission-associated proteins were decreased and Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activity was decreased compared with control rats (59,60). ATPase activity was decreased to varying degrees in this rat model, leading to a decrease in ATP synthesis and inability of muscles to complete contraction and diastolic movement. Ke *et al* (33) suggested that the mitochondrial biogenesis signaling pathway is associated with MG, verifying the association between MG and mitochondrial dynamics and biogenesis.

In the present study, expression levels of fusion-associated proteins Mfn1/2 and Opa1 and fission-associated proteins Fis1 and Drp1 were significantly lower in patients with MG than in control subjects. However, the mRNA expression levels of Mfn1, Mfn2 and Opa1 decreased, while those of Fis1 and Drp1 increased in patients with MG compared with control subjects. Both the protein and mRNA expression levels of mitochondrial biogenesis-associated factors AMPK, PGC-1α, NRF-1 and TFAM were decreased in PBMCs of patients with MG. Of 50 patients with MG included in the present study, 6 patients were anti-AChR-negative. Gene expression analysis in these patients (anti-AChR-negative) demonstrated that mRNA expression level was consistent with the whole sample (including anti-AChR-negative and -positive) result, indicating that this subset of patients also have mitochondrial energy metabolism decreased.

ROC curve analysis showed that mitochondrial dynamics- and biogenesis-associated factors were specific and sensitive for diagnosing MG. Excluding AMPK, AUC values of the Mfn1/2, Opa1, Fis1, Drp1, PGC-1α, NRF-1 and TFAM were 0.6212-0.8696 ($P<0.05$). Furthermore, the AUC values of PGC-1α, NRF-1 and TFAM were >0.8. A higher AUC value indicates a greater potential to distinguish patients from controls. Thus, ROC curve analysis indicated that these proteins may serve as promising biomarkers for MG. However, the present study had limitations. In the absence of clinical diagnosis, it is difficult to distinguish MG from other types of mitochondria-associated disease based on levels of mitochondrial indicators. In addition, the present study only collected patients with type IIb MG and obtained AUC>0.8, but this result is not comprehensive. The present study did not determine the explicit mechanism that how suffering MG underlying mitochondrial function disorder and to distinguish MG from other types of mitochondria-associated disease. In future, the potential diagnostic value of mitochondria-associated indicators in MG should be further researched.

In conclusion, expression levels of mitochondrial dynamics- and biogenesis-associated factors in PBMCs were significantly different between patients with MG and control subjects. These factors may serve as potential diagnostic biomarkers for MG.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YS conceived and designed the experiments. LL, DC and HZ performed most of experiments and drafted the manuscript. JLiang, AJ and FL performed the formal analysis and interpretation of data. YS provided funding and gave final approval of the version to be published. JLi, ZC, QJ and PL carried out part of the PCR experiments and collected all clinical data. JS and WJ performed isolation of PBMCs and revised the manuscript from a critical perspective for important intellectual content. QL and LK performed the statistical analysis, confirmed the authenticity of all the raw data and gave final approval of the version to be published. JS and WJ agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All participants understood the experimental procedure and provided written informed consent. Written informed consent was obtained from parents/guardians of all participants <18 years old. All procedures involving human subjects were approved by the Academic Ethics Committee of The First Affiliated Hospital of Guangzhou University of Chinese Medicine [approval no. ZYYZCK(2018)075].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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