Proteomic profiling of the vastus lateralis muscles from dysferlinopathy patients

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Written by Hyung Jun Park

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ABSTRACT

Proteomic profiling of the vastus lateralis muscles from dysferlinopathy patients

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Dysferlinopathy is an autosomal recessive disease caused by a *DYSF* gene mutation and one of the most common limb-girdle muscular dystrophies in Korea. We compared muscle protein extracts from dysferlinopathy patients and healthy controls to identify new biomarkers of this myopathy. We reviewed the medical records of the myopathy database at Gangnam severance hospital from January 2002 to October 2016. Eight vastus lateralis muscle samples from five dysferlinopathy patients and three control subjects were selected. We identified control subjects with the following criteria: (i) normal muscle pathology, (ii) normal serum creatine kinase level, (iii) no definite muscle weakness, and (iv) available vastus lateralis muscle biopsy samples. We separated proteins/peptides from these eight muscle specimens using two-dimensional electrophoresis (2DE). Data were acquired from liquid chromatography-mass spectrometry protein fragmentation patterns after comparing the spot volumes. Western blotting revealed total dysferlin loss in the dysferlinopathy patients but normal expression in the control subjects. 2DE indicated somewhat diverse protein constellations between the dysferlinopathy and control groups. For comparisons of spot volumes, image analysis was carried out for two dysferlinopathy samples and one control sample. We selected 88 spots from the two dysferlinopathy patients that were either more than twice or less than half of the

volumes of those in the muscles from the control subject. We then selected 44 spots with volumes that were consistently different between all samples from two groups. Liquid chromatography-mass spectrometry indicated 26 differently expressed proteins. Western blotting revealed that creatine kinase M-type, carbonic anhydrase III (muscle specific) and desmin were significantly elevated in dysferlinopathy muscle. These results confirm the usefulness of the classic biomarker and have newly identified the altered expression of proteins in the vastus lateralis muscles of dysferlinopathy patients.

Key Words: dysferlinopathy; electrophoresis, gel, two-dimensional; mass spectrometry; proteomics

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I. INTRODUCTION

Genetic myopathy is a clinically and genetically heterogeneous group of genetic disorders characterized by progressive degeneration of skeletal muscles. More than 200 causative genes have now been identified for genetic myopathies.[1] Promising therapeutic approaches for these conditions include restoring dystrophin gene expression via exon-skipping strategies, viral-based gene therapies, and read-through strategies.[2-4] There is an urgent need therefore for a reliable surrogate biomarker or set of biomarkers for genetic myopathy, ideally based on readily accessible and measurable molecules.[5] Proteomic profiling is the most powerful tool for the fast identification of novel biomarkers that are involved in these disorders. Two-dimensional gel electrophoresis (2-DE) is frequently used as the separation strategy for the proteomic evaluation of soluble components.[6] The use of mass spectrometry for the large-scale screening of proteins also strongly enhances the research capabilities of the modern biochemical laboratory.[7] However, almost all proteomic analysis of genetic myopathy to date has focused on dystrophinopathy, which is the most commonly studied genetic myopathy.[8,9]

Dysferlinopathy is an autosomal recessive disease caused by a *DYSF* (MIM*603009) gene mutation, and is one of most common genetic myopathies found in Korea.[10] The *DYSF* gene encodes dysferlin, a 230 kDa protein with

seven C2 domains and a single transmembrane domain at the C terminus. Dysferlin is homologous to the *Caenorhabditis elegans* spermatogenesis factor fer-1 protein that mediates the fusion of intracellular vesicles to the sperm plasma membrane. It has been suggested that dysferlin might be a vesicle-associated membrane protein involved in the docking and fusion of vesicles in muscle cells.[11] Hence, dysferlin has multiple roles during the process of membrane repair, myoblast differentiation, tubulogenesis and muscle regeneration. Clinically, dysferlinopathy has various clinical presentations such as Miyoshi distal myopathy, limb-girdle muscle weakness, mixed proximodistal weakness, and distal anterior compartment weakness.[12] This clinical phenotype of dysferlinopathy is distinguishable from that of dystrophinopathy. These clinical features suggest the possibility of novel biomarker discovery in dysferlinopathy. However, there has been only one study which investigated proteomic profiling of skeletal muscles in dysferlinopathy.[13]

In our current study therefore, we aimed to further identify new biomarkers of dysferlinopathy by comparing muscle proteins from affected patients and healthy controls.

II. MATERIALS AND METHODS

1. Study subjects

We reviewed medical records of the myopathy database from January 2002 to October 2016. The study hospital is Gangnam Severance hospital, which is one of the largest myology centers in Korea, and our database contained 727 unrelated patients with genetic myopathy. Five vastus lateralis muscle samples from five patients with dysferlinopathy (MF635, MF407, MF266, MF524, and MF558) and three control subjects (MF592, MF1046, and MF1213) were enrolled in this study. The dysferlinopathy cases were genetically confirmed. For the selection of control subjects, we identified three individuals who fulfilled the following criteria: (i) normal muscle pathology, (ii) normal serum

creatine kinase level, (iii) no definite muscle weakness, and (iv) available vastus lateralis muscle biopsy samples. The institutional review board of Gangnam Severance hospital, Korea, approved the research protocol (IRB No. 3-2016-0335).

2. Sample preparation and two-dimensional electrophoresis (2DE)

Sample preparation and 2DE were performed as described by Bahk et al.[14] Briefly, the frozen vastus lateralis muscles of five dysferlinopathy and three controls subjects were powdered under liquid nitrogen using a mortar and pestle and suspended in 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2.5% (w/v) DTT and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA). The lysates were centrifuged at 15,000 × g for 20 min and suitably stored at -80℃. Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA, USA). For 2DE analysis, pH 3-10 immobilized pH gradient (IPG) strips (GE Healthcare Life Sciences, Pittsburgh, PA, USA) were rehydrated in swelling buffer containing 7 M urea, 2 M thiourea, 2.5% (w/v) DTT, and 4% (w/v) CHAPS. The protein lysates (800 μg) were loaded into the rehydrated IPG strips using an IPG phor III (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and the 2D separation was performed on 14% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Following fixation of the gels for 1 h in a solution of 40% (v/v) methanol containing 5% (v/v) phosphoric acid, the gels were stained with Colloidal Coomassie Blue G-250 solution (ProteomeTech, Seoul, Korea). The gels were destained using deionized water and images were acquired with image scanner (Bio-Rad, Hercules, CA, USA). Image analysis was carried out using ImageMaster™ 2D Platinum software (Amersham Biosciences, Hercules, CA, USA). More than 25 spots in all gels were correspondingly landmarked and normalized to enable comparisons. For comparisons of spot volumes, image analysis was carried out for two dysferlinopathy samples and one control sample. We selected spots

from the two dysferlinopathy patients that were either more than twice or less than half of the volumes of those in the muscles from the control subject. We then selected spots with volumes that were consistently different between all samples from two groups.

3. In-gel tryptic digestion and peptide extraction

Forty-four differentially expressed spots were excised and digested from the 2DE gels. The procedure for in-gel digestion of protein spots from Coomassie Blue stained gels was carried out as described previoously.[14] Briefly, protein spots were excised from the stained gels and cut into pieces. The gel pieces were washed for 1 h at room temperature in 25 mM ammonium bicarbonate (ABC) buffer, pH 7.8, containing 50 % (v/v) acetonitrile. Following the subsequent dehydration of the pieces in a centrifugal vacuum concentrator for 10 min, they were rehydrated in 50 ng of sequencing grade trypsin solution (Promega, Madison, WI, USA). After incubation in 25 mM ABC buffer, pH 7.8, at 37℃ overnight, the tryptic peptides were extracted with 5 μl of 1% formic acid containing 50% (v/v) acetonitrile for 40 min with mild sonication. The extracted solution was concentrated using a centrifugal vacuum concentrator.

Prior to mass spectrometric analysis, the peptide solution was subjected to a desalting process using a reversed-phase column.[15] Briefly, after an equilibration step with 10 μl of 5% (v/v) formic acid, the solution was loaded onto the column and washed with 10 μl of 5% (v/v) formic acid. The bound peptides were then eluted with 5 μl of 70% acetonitrile with 5% (v/v) formic acid.

4. Identification of proteins by liquid chromatography tandem-mass spectrometry (LC-MS/MS)

LC–MS/MS analysis was performed using Waters Nano ultra-performance liquid chromatography and an LTQ-orbitrap-mass spectrometer (Thermo

Electron, San Jose, CA). The capillary column used for this LC–MS/MS analysis (150 mm × 0.075 mm) was obtained from Proxeon (Odense M, Denmark) and was packed in-house with a 5 μm, 100 Å pore size Magic C18 stationary phase slurry (Michrom Bioresources, Auburn, CA). Mobile phase A for the liquid chromatography separation was a 0.1% formic acid solution in deionized water and mobile phase B comprised 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 6% B to 50% B for 22 min, from 50% B to 95% B for 5 min, and from 95% B to 6% B for 13 min. The flow rate was 0.4 uL/min.

For tandem mass spectrometry, the mass spectra were acquired using data-dependent acquisition with a full mass scan (350-1800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscan on the LTQ. The temperature of the ion transfer tube was controlled at 200℃ and the spray was 1.5-2.0 kV. The normalized collision energy was set at 35% for MS/MS. The individual spectra from MS/MS were processed using SEQUEST software (Thermo Quest, San Jose, CA, USA) and the generated peak lists were used to query our in-house database using the MASCOT program (Matrix Science Ltd., London, UK). We set the modifications of methionine, and cysteine, methylation of arginine, and phosphorylation of serine, threonine, and tyrosine for MS analysis and a peptide mass tolerance of 2 Da. The MS/MS ion mass tolerance was 1 Da and the allowance of missed cleavage was 1. Charge states (+1, +2, +3) were taken into account during the data analysis. We took only significant hits as defined by MASCOT probability analysis.

5. Western blotting

For Western blotting analysis, a commercial Protein Assay Dye Reagent (Bio-Rad) was first used to quantify the lysates. Thirty micrograms of protein from each sample were then resolved on 12 well Precast 4% to 12% gels (Komabiotech) and transferred to PVDF membranes (Amersham Biosciences).

The membranes were blocked for 30 min in Tris-buffered saline (TBS) containing 0.1% tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with primary antibodies against one of the following proteins: dysferlin (Leica Biosystems, Newcastle Upon Tyne, UK); myotilin isoform β; creatine kinase M-type; ATP synthase subunit alpha, mitochondrial isoform a precursor; ATP synthase subunit beta, mitochondrial precursor; carbonic anhydrase isoform 3, muscle specific; voltage-dependent anion-selective channel protein 2; 14-3-3 protein **δ/ζ**; heat shock 70k Da protein 1B; heat shock protein β-1; α-actin; troponin I; very long-chain specific acyl-CoA dehydrogenase, mitochondrial; glyceraldehyde 3-phosphate dehydrogenase; enolase 3; myoglobin (all from Santa Cruz Biotechnology, Santa Cruz, CA, US); α-tubulin; β-tubulin; desmin; aconitase 2, mitochondrial; lamin A/C; myosin regulatory light chain 2, skeletal muscle isoform; vimentin; pyruvate kinase isozyme PKM isoform b (all from Cell Signaling Technology, Danvers, MA, USA); ankyrin repeat domain 2; eukaryotic translation initiation factor 5A-1; myosin light chain 1/3 (all from Abcam, Cambridge, MA, USA); and delta (3,5)-delta (2,4)-dienoyl-CoA isomerase, mitochondrial precursor (Sigma-Aldrich, St Louis, MO, US). The membranes were then washed with TBS-0.1% tween 20, incubated for 1 h with a secondary antibody, and visualized using the ECL detection kit (AB Frontier Co., Ltd., Seoul, Korea).

6. Statistical analysis

The Mann-Whitney test was used to compare the volume ratio of each protein over that of glyceraldehyde 3-phosphate dehydrogenase between the dysferlinopathy samples and controls. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were conducted using R 3.1.2 (www.r-project.org).

III. RESULTS

Western blotting for dysferlin revealed a total loss of protein expression in the five dysferlinopathy study patients but normal expression in the three control subjects (Figure 1).

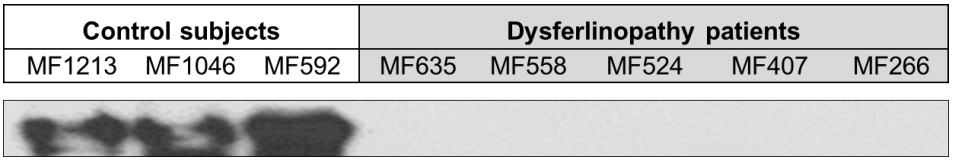

Figure 1. Dysferlin band pattern on western blotting. Western blotting for dysferlin revealed normal expression in three control subjects yet total loss in five dysferlinopathy patients.

Three of these patients (MF407, MF266, and MF524) were initially diagnosed with Miyoshi distal myopathy and two (MF635 and MF558) with limb-girdle muscular dystrophy 2B. When muscle biopsies were performed, two patients (MF635 and MF407) were in their early 20s and were mildly affected. However, three patients (MF266, MF524, and MF558) were in their mid-30s to early 40s and were moderately affected. Among control subjects, two subjects (MF592 and MF1213) were finally diagnosed with psychogenic weakness and one (MF1046) with muscle cramp. Table 1 summarizes the clinical, laboratory and pathological spectrum of these patient subjects.

Table1. Clinical presentations of the dysferlinopathy patients and control subjects

Subject number	Sex	Age at Dx, year	*DYSF* mutations	Initial presentation	GMW score	CK, IU/L
MF635	F	19	c.2494C>T + c.2974T>C	LGMW	3	18,380
MF407	M	22	c.663+1G>C + c.1284+2T>C	DW	2	10,870
MF266	F	41	c.1579G>T + c.3406delG	DW	5	5,786
MF524	F	36	c.663+1G>C + c.2494C>T	DW	5	-
MF558	F	38	c.2964C>A + c.4434G>A	LGMW	4	8,153
MF592	F	16	-	Psychogenic weakness	-	54
MF1046	F	47	-	Cramp	-	34
MF1213	M	14	-	Psychogenic weakness	-	137

Abbreviation: F, female; M, male; Dx, diagnosis; GMW, modified Gardner-Medwin and Walton score; CK, creatine kinase; IU/L, international unit per liter; GMW, limb-girdle muscle weakness; DW, distal muscle weakness; and VL, vastus lateralis muscle

Figure 2 shows 2D-electrophoresis gel of a vastus lateralis muscles protein extract from five dysferlinopathy patients and three control subjects.

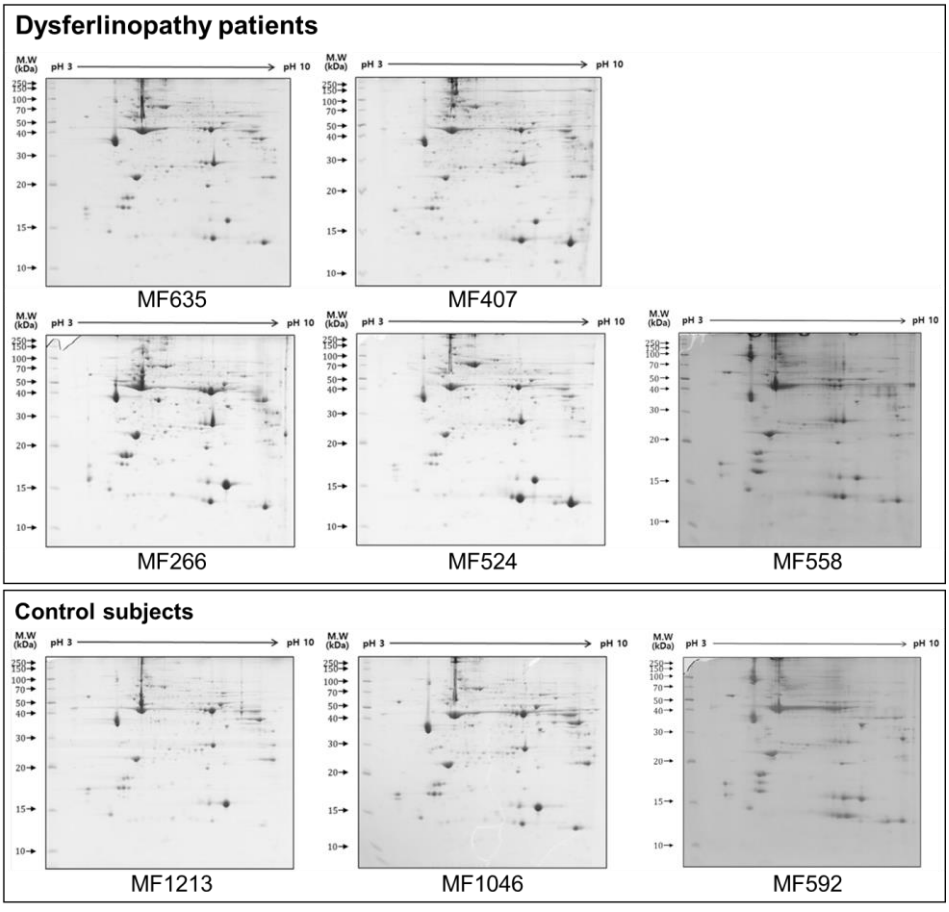

Figure 2. Two-dimensional electrophoresis images of the vastus lateralis protein extract from dysferlinopathy patients and control subjects.

For comparison of spot volumes, image analysis was carried out in the skeletal muscle from one control subject (MF592) and those from two dysferlinopathy patients (MF635 and MF266) (Figure 3).

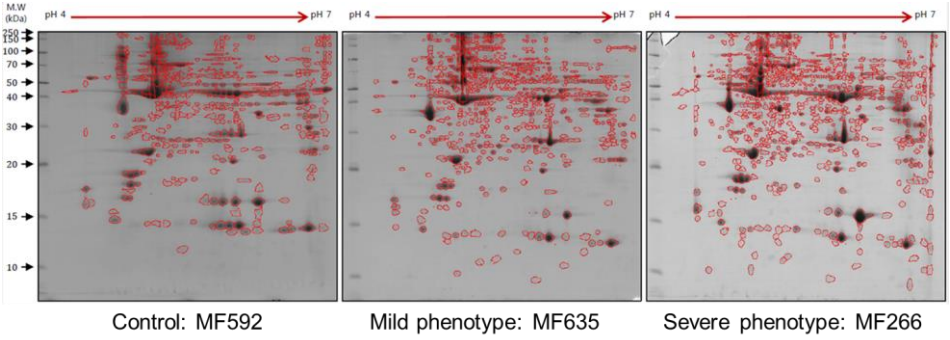

Figure 3. Image analysis of three typical samples. Image analysis was carried out using ImageMaster™ 2D Platinum software (Amersham Biosciences, Hercules, CA, USA). For the comparison of protein spots, more than 25 spots in all gels were correspondingly landmarked and normalized (MF592, control; MF635, mild phenotype; MF266, severe phenotype).

Eighty spots were found to be differently expressed between the control and dysferlinopathy samples, with 44 upregulated spot volumes and 36 downregulated volumes identified in the skeletal muscles from the dysferlinopathy subjects. We selected 44 spots with consistently different volumes between the samples. Protein fragmentation patterns of these spots were evaluated by liquid chromatography-mass spectrometry and identified 26 differently expressed proteins (Table 2).

Table 2. Differentially expressed protein. Accession numbers correspond to the NCBI BLAST and the Swiss-Prot database.

Protein name	NCBI BLAST	Swiss-Prot accession number	pI	Mw (Da)
MYOT	NP_001129412.1	Q9UBF9	9.18	55,827
CKM	NP_001815.2	P06732	6.77	43,330
ATP5F1A	NP_004037.1	P25705	9.16	59,868
ATP5F1B	NP_001677.2	P06576	5.26	56,557
CA3	AAH04897.1	P07451	6.86	29,843
VDAC2	CAG33245.1	P45880	7.49	32,082
TUBA1A	CAA30026.1	P68366	4.95	50,665
TUBB	AAH20946.1	P07437	4.78	50,136
YWHAZ	NP_003397.1	P63104	4.63	29,346
HSPA1B	NP_005337.2	Q27965	5.48	70,350
HSPB1	NP_001531.1	P04792	5.98	22,834
MYL1	CAB42646.1	P05976	4.97	21,206
MYLPF	NP_037424.2	Q96A32	4.91	19,130
DES	AAC39938.1	P17661	5.21	53,611
ACO2	BAG37362.1	Q99798	7.36	86,176
ACTA1	NP_001091.1	P68133	5.23	42,389
LMNA	EAW52998.1	P02545	6.57	74,440
TNNI2	NP_003273.1	P48788	8.87	21,509
ACADVL	BAG57027.1	P49748	5.92	71,354
ANKRD2	CAC19411.1	Q9GZV1	5.71	40,031
EIF5A	NP_001961.1	P63241	5.08	17,058
PKM	NP_872270.1	P14618	7.96	58,502
VIM	NP_003371.2	P08670	5.06	53,731
ECH1	NP_001389.2	Q13011	8.16	36,161
ENO3	AAH17249.1	Q27727	7.59	47,335
MB	AAA59595.1	P02144	7.14	17240

Abbreviation: pI, isoelectric point; Mw, molecular weight; MYOT, myotilin; CKM, creatine kinase M-type; ATP5F1A, ATP synthase subunit alpha, mitochondrial isoform a precursor; ATP5F1B, ATP synthase subunit beta, mitochondrial precursor; CA3, carbonic anhydrase isoform 3, muscle specific; VDAC2, voltage-dependent anion-selective channel protein 2; TUBA1A, α-tubulin 1A chain; TUBB, β-tubulin; YWHAZ, 14-3-3 protein **δ/ζ;** HSPA1B, heat shock 70kDa protein 1B; HSPB1, Heat shock protein β-1; MYL1, Myosin light chain 1/3, skeletal muscle isoform; MYLPF, myosin regulatory light chain 2, skeletal muscle isoform; DES, desmin; ACO2, aconitase 2, mitochondrial; ACTA1, α-actin, skeletal muscle; LMNA, lamin A/C; TNNI2, troponin I fast skeletal muscle isoform 1; ACADVL, very long-chain specific acyl-CoA dehydrogenase, mitochondrial; ANKRD2, ankyrin repeat domain 2; EIF5A,

eukaryotic translation initiation factor 5A-1; PKM, pyruvate kinase isozyme PKM isoform b; VIM, vimentin; ECH1, delta (3,5)-delta (2,4)-dienoyl-CoA isomerase, mitochondrial precursor; ENO3, enolase3; and MB, myoglobin

Further Western blotting analysis verified 21 of these proteins but five proteins (myoglobin; delta (3,5)-delta (2,4)-dienoyl-CoA isomerase, mitochondrial precursor; vimentin; enolase3; and pyruvate kinase isozyme PKM isoform b) were not detected in any of the eight muscle samples (Figure 4).

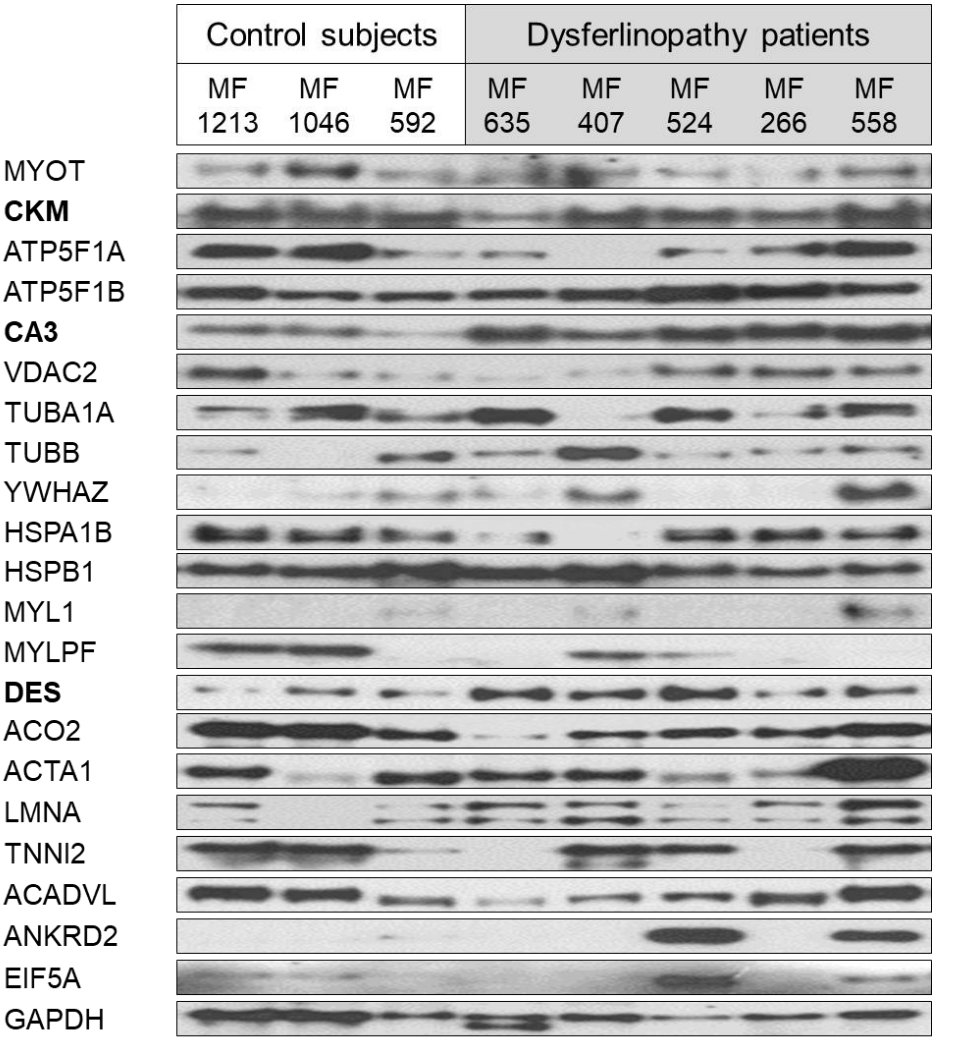

Figure 4. Detection of 21 selected proteins and housekeeping control (glyceraldehyde 3-phosphate dehydrogenase) by Western blotting. Abbreviation: GAPDH, glyceraldehyde 3-phosptage dehydrogenase. Abbreviation: MYOT, myotilin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CKM, creatine kinase M-type; ATP5F1A, ATP synthase subunit alpha, mitochondrial isoform a precursor; ATP5F1B, ATP synthase subunit beta, mitochondrial precursor; CA3, carbonic anhydrase isoform 3, muscle specific; VDAC2, voltage-dependent anion-selective channel protein 2; TUBA1A, α-tubulin 1A chain; TUBB, β-tubulin; YWHAZ, 14-3-3 protein **δ/ζ;** HSPA1B, heat shock 70kDa protein 1B; HSPB1, Heat shock protein β-1; MYL1, Myosin light chain 1/3, skeletal muscle isoform; MYLPF, myosin regulatory light chain 2, skeletal muscle isoform; DES, desmin; ACO2, aconitase 2, mitochondrial; ACTA1, α-actin, skeletal muscle; LMNA, lamin A/C; TNNI2, troponin I fast skeletal muscle isoform 1; ACADVL, very long-chain specific acyl-CoA dehydrogenase, mitochondrial; ANKRD2, ankyrin repeat domain 2; and EIF5A, eukaryotic translation initiation factor 5A-1

Table 3 showed the volume ratio of each protein over glyceraldehyde 3-phosphate dehydrogenase between the dysferlinopathy samples and controls. Three proteins (creatine kinase M-type; carbonic anhydrase III, muscle specific; and desmin) were significantly elevated in the muscle samples from the dysferlinopathy patients. Additionally, four proteins (myosin light chain 1/3, skeletal muscle isoform; lamin A/C; ankyrin repeat domain 2; and eukaryotic translation initiation factor 5A-1) were inconsistently elevated in the muscle samples from the dysferlinopathy patients.

Table 3. Volume ratio of each protein compared to a glyceraldehyde 3-phosphate dehydrogenase housekeeping control

Ratio of each protein over GAPDH	Control subjects			Dysferlinopathy patients					p-value
	MF 1213	MF 1046	MF 592	MF 635	MF 407	MF 524	MF 266	MF 558	
MYOT	0.24	0.42	0.35	3.29	0.41	0.37	0.90	0.49	0.101
CKM	0.51	0.48	0.76	3.47	0.78	1.15	3.60	1.56	0.025
ATP5F1A	0.52	0.67	0.29	1.37	0.02	0.31	2.38	1.19	0.456
ATP5F1B	0.57	0.63	3.75	0.52	1.11	5.19	1.42	1.41	0.655
CA3	0.43	0.47	0.26	8.81	0.74	1.87	6.48	2.15	0.025
VDAC2	0.40	0.19	0.79	0.05	0.14	1.74	0.60	0.65	0.881
TUBA1A	0.29	0.79	0.75	8.67	0.09	1.36	1.78	1.23	0.180
TUBB	0.10	0.05	2.91	0.19	0.97	0.71	0.24	0.65	0.456
YWHAZ	0.07	0.18	2.50	0.20	0.77	0.46	0.14	1.85	0.655
HSPA1B	0.66	0.58	0.63	1.77	0.04	1.07	3.56	1.00	0.180
HSPB1	0.53	0.86	7.66	0.78	1.57	3.57	0.88	1.45	0.655
MYL1	0.04	0.07	0.07	0.05	0.12	0.24	0.07	0.35	0.115
MYLPF	0.48	0.57	0.11	0.56	0.42	0.31	0.38	0.06	0.456
DES	0.07	0.20	0.24	3.87	0.39	0.84	1.09	0.52	0.025
ACO2	1.01	1.11	1.00	0.91	0.57	1.15	3.76	1.84	0.655
ACTA1	0.67	0.29	6.84	0.72	1.19	1.94	0.63	4.98	0.655
LMNA	0.26	0.05	0.85	0.65	1.22	1.08	0.54	2.54	0.101
TNNI2	0.96	1.06	0.27	0.49	1.15	1.25	0.32	1.60	0.297
ACADVL	0.59	0.53	0.45	1.04	0.26	0.56	2.84	1.25	0.297
ANKRD2	0.05	0.08	0.17	0.08	0.12	5.41	0.13	1.64	0.154
EIF5A	0.30	0.24	0.31	1.84	0.30	0.83	0.87	0.38	0.072

Abbreviation: MYOT, myotilin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CKM, creatine kinase M-type; ATP5F1A, ATP synthase subunit alpha, mitochondrial isoform a precursor; ATP5F1B, ATP synthase subunit beta, mitochondrial precursor; CA3, carbonic anhydrase isoform 3, muscle specific; VDAC2, voltage-dependent anion-selective channel protein 2; TUBA1A, α-tubulin 1A chain; TUBB, β-tubulin; YWHAZ, 14-3-3 protein **δ/ζ;** HSPA1B, heat shock 70kDa protein 1B; HSPB1, Heat shock protein β-1; MYL1, Myosin

light chain 1/3, skeletal muscle isoform; MYLPF, myosin regulatory light chain 2, skeletal muscle isoform; DES, desmin; ACO2, aconitase 2, mitochondrial; ACTA1, α-actin, skeletal muscle; LMNA, lamin A/C; TNNI2, troponin I fast skeletal muscle isoform 1; ACADVL, very long-chain specific acyl-CoA dehydrogenase, mitochondrial; ANKRD2, ankyrin repeat domain 2; and EIF5A, eukaryotic translation initiation factor 5A-1

IV. DISCUSSION

We demonstrated 26 differently expressed proteins using the 2DE and LC-MS/MS methods. This number of identified proteins was similar to 29 proteins of previous study.[13] However, there were only two differently expressed proteins (myosin regulatory light chain 2, skeletal muscle isoform and α-actin, skeletal muscle) in both studies. We found no differences in the expression of α-actin, alpha skeletal muscle; heat shock protein beta-1; or myosin regulatory light chain 2, skeletal muscle isoform, which were reported to be differently expressed in the previous proteomic study of dysferlinopathy.[13] Additionally, the protein constellation patterns were also found to be highly diverse in our current analyses. This is likely because skeletal muscle is highly heterogeneous and contains multinucleated slow-oxidative, fast-glycolytic, or hybrid cell types, in conjunction with satellite cell pools underneath the basal lamina, distinct layers of connective tissue comprising the endomysium, perimysium and epimysium, a complex network of capillaries, and motor neurons and neuromuscular junctions.[16,17] Therefore, we analyzed the volume of spots in three samples with many spots and then compared the different volume of spots between dysferlinopathy and control groups.

We have identified a significantly elevated expression of three proteins (creatine kinase M-type, carbonic anhydrase isoform 3, and desmin) in the vastus lateralis muscles of dysferlinopathy patients compared with normal controls. These proteins were not described in a previous proteomic study of dysferlinopathy.[13] These proteins are closely associated with skeletal muscle disorders however. Creatine kinase M-type catalyzes the conversion of creatine

and utilizes adenosine triphosphate to produce phosphocreatine and adenosine diphosphate. It is a well-known protein that is elevated in the skeletal muscle and serum from patients with genetic myopathy, especially dysferlinopathy.[12,18] Carbonic anhydrase is part of a family of metalloenzymes that catalyze the reversible hydration of carbon dioxide and is found at the highest levels among these family members in skeletal muscle.[19] This protein is also one of the general markers of skeletal muscle damage.[20] Additionally, a previous study has reported that carbonic anhydrase isoform 3 is highly expressed in senescent skeletal muscle.[21] Desmin is a muscle specific protein that integrates the sarcolemma, Z disk, and nuclear membrane in sarcomeres and regulates the sarcomere architecture.[22] Alterations to this protein result in desmin-related myofibrillar myopathy.[23] Additionally, four proteins (myosin light chain 1/3, skeletal muscle isoform; lamin A/C; ankyrin repeat domain 2; and eukaryotic translation initiation factor 5A-1) showed inconsistently elevated expression in the muscle samples from the dysferlinopathy patients. Among them, lamin A/C is one of the causative protein of Emery-Dreifuss muscular dystrophy.[24]

This study had some noteworthy limitations, including the limited number of muscle samples from dysferlinopathy and control subjects. Additionally, we could not match the patients and control subjects based on their age and gender. However, muscle specimens cannot be readily obtained from healthy subjects because of the invasiveness of the procedures. To overcome this limitation, we used only the vastus lateralis muscles from our myopathy cases and control subjects. We also selected control subjects with a normal serum CK level, normal muscle pathology, and no definite motor weakness.

V. CONCLUSION

The present study revealed several differentially expressed proteins in the vastus lateralis muscles from dysferlinopathy patients. However, the application of these proteins as biomarkers is limited because that invasive muscle biopsy is

needed. Therefore, further studies are needed to assess the differentially expression of these proteins in serum. In conclusion, we confirm the usefulness of the classic biomarker and have newly identified the altered expression of proteins in the vastus lateralis muscles of dysferlinopathy patients, which provides new insights into identifying biomarkers of genetic myopathy.

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ABSTRACT (IN KOREAN)

디스펄린병 환자 유래 가쪽넓은근의 단백질체 프로파일링

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박형준

디스펄린병은 *DYSF* 유전자의 돌연변이로 발생하는 상염색체열성 유전성 근육질환으로 한국에서 가장 많은 팔다리이음근디스트로피 중 하나이다. 본 연구는 유전성 근육병의 새로운 생체 지표를 찾기 위해서 디스펄린병 환자와 대조군의 근육조직에서 단백추출물의 차이를 비교하였다. 2002년 1월부터 2016년 10월까지 강남세브란스 병원을 방문한 근육병환자의 의무기록을 조사하여 대상 환자를 찾았고 근육병 데이터베이스에서 근육 검체의 유무를 확인하였다. 최종적으로 5명의 증 환자와 3명의 대조군에서 가쪽넓은근을 확인하여 이를 대상으로 연구를 수행하였다. 5명의 디스펄린병 환자들은 모두 유전자검사에서 돌연변이가 확인된 환자였다. 8개의 가쪽넓은근에서 2차원전기영동을 수행하였다. 이미지 분석을 위해서 점이 가장 많은 디스펄린병 환자의 근육 2개와 대조군 근육 1개를 선별한 후 단백의 양이 2배이상이거나 1/2이하인 점들을 찾았다. 그 후 해당 점들이 다른 근육들에서도 차별적으로 발현한 점을 선택하였다. 선별된 점에서 액체크로마토그래피와 진량분광분석을 통해서 단백파쇄양상의 데이터를 얻었다. 디스펄린에 대한 웨스턴블럿에서 3명의 디스펄린병 환자의 근육에서는 단백이 전혀

발현되지 않고 대조군에서는 정상적으로 발현되었다. 2차원전기영동 후 단백에 대한 점들의 분포는 디스펄린병군과 대조군 모두에서 매우 다양하였다. 점의 수가 많고 또렷했던 디스펄린병 환자(MF635 와 MF266)의 근육 2개와 대조군(MF592)의 근육 1개를 비교할 때 총 80개의 점에서 발현 차이가 있었다. 각 점들을 다른 대조군과 환자군의 2차원전기영동의 결과와 비교하여 이 중 44개의 점들에서 일관된 발현 차이가 있음을 확인하였다. 액체크로마토그래피와 질량분광분석을 통해서 44개의 점들에서 26개의 단백을 확인하였다. 26개의 단백에 대해서 웨스턴블럿으로 단백 발현을 다시 정량하였을 때 21개의 단백은 확인되었으나 5개의 단백들(myoglobin과 delta (3,5)-delta (2,4)-dienoyl-CoA isomerase, mitochondrial precursor과 vimentin과 enolase3과 pyruvate kinase isozyme PKM isoform b)은 확인되지 않았다. glyceraldehyde 3-phosphate dehydrogenase의 양을 기준으로 단백량을 비교할 때 특히 3개의 단백(creatine kinase M-type과 carbonic anhydrase III, muscle specific과 desmin)이 디스펄린병 환자에서 의미 있게 발현이 높았다. 결론적으로 본 연구는 디스펄린병 환자의 가쪽넓은근에서 단백의 발현 변화를 보여주었다. 본 연구 결과는 디스펄린병의 생체 지표를 규명하기 위한 새로운 통찰을 제공할 것으로 생각된다.

핵심되는 말: 디스펄린병; 이차원전기영동, 질량분광분석, 단백질체학

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