が、N–アセチルシステインの添加により細胞内 ROS 濃度は低下した。血清および NeuAc 添加により、Menadione による ROS 産生は抑制された。

一方、H_2O_2 による ROS 産生では、DMRV 由来細胞はコントロールマウス由来細胞よりも高かった。血清および NeuAc 添加により、H_2O_2 による ROS 産生は抑制されたが、高濃度の H_2O_2 には効果をしめさなかった。

D. 考察

筋ジストロフィーモデルマウスやミオパチーモデルで、病態への ROS の関与が提案されている。抗酸化薬の投与試験では、N–アセチルシステインは筋萎縮に対して効果を示し、また筋力も回復した。このことは、DMRV における GNE 遺伝子の変異にともなう低シアル酸が引き起こすミオパチーに関して、ROS が発症機序に重要は役割を示すこと、筋萎縮のみならず、筋力低下にも関わることが考えられた。

昨年度、DMRV マウスでは、加齢に伴い、筋収縮に伴うヒドロキシラジカルの量が増加することを報告した。このことから、2つの可能性が考えられた。一つは、DMRV マウス骨格筋では ROS の生成量が増大しているというもの、もう一つは ROS の除去能力が減少しているというものである。今年度の解析では、DMRV マウスの初代骨格筋細胞において、ROS の除去能力が低下していることが、示された。DMRV マウスで観察される ROS の除去の特異的減少は、H_2O_2 によるヒドロキシラジカルでは観察されたものの、Menadione による O_2 ラジカル産生に対しては観察されなかった。このことは、DMRV マウスでは、ヒドロキシラジカルに対する除去能力が特異的に低下していること、また、ヒドロキシラジカルが DMRV の病態形成に関わっている可能性が示された。マウスにおける in vivo でのヒドロキシラジカルの上昇と関連しているものと思われた。面白いことに、シアル酸の添加および N–アセチルシステインの添加では、細胞内のヒドロキシラジカルおよび O_2 ラジカル産生に対して、同様に除去効果を示した。

E. 結論

DMRV モデルマウスにおける筋萎縮と筋力低下への ROS の関連が示唆された。ヒドロキシラジカルの除去効果の低下が、病態と関連する可能性が示された。

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総説

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G. 知的財産権の出願・登録状況
（予定を含む）

1. 特許申請中

特許の名称：GNE タンパク質の機能低下に起因する疾患の治療用医薬剤、食品組成物、食品添加物
発明者名：
野口　悟，Malicdan MC，西野一三
権利者名：
財団法人ヒューマンサイエンス振興財団
特許の種類：特許
番号：特願 2011-513374
出願日：20090515
国内外別：国際

特許の名称：タンパク質蓄積性筋疾患を治療するための医薬
発明者名：
野口　悟，Malicdan MC，西野一三
権利者名：野口　悟
特許の種類：特許
番号：特願 2011-042435
出願日：20110228
国内外別：国内

2. 実用新案登録

特になし

3. その他

特になし

厚生労働科学研究費補助金（障害者対策総合研究事業（神経・筋疾患分野））
分担研究報告書

縁取り空胞を伴う遠位型ミオパチーの治療効果最大化のための研究

～三好型遠位型ミオパチーの自然経過に関する研究～

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研究要旨　三好型を中心として他の遠位型ミオパチーの実態に関する情報収集を行う。三好型遠位型ミオパチーの特徴を明らかにするため、dysferlin 遺伝子変異の特徴を解析した。変異は遺伝子全体に広く分布し４種類の日本人に多い変異があった。三好型遠位型ミオパチーに特有の変異が存在した。本研究は将来 dysferlinopathy の治療研究がおこなわれる際の重要な基礎資料となると考える。

A. 研究目的

Dysferlinは1998年三好型遠位型ミオパチーの原因遺伝子としてクローニングされた。肢帯型筋ジストロフィー2B型の原因であることも判明し、dysferlinopathyという概念が確立した。我々は日本人三好型遠位型ミオパチーおよび肢帯型筋ジストロフィーにおいて同遺伝子異常のスクリーニングを行ってきた。Polymerase chain reaction (PCR)-Single strand conformation polymorphism (SSCP) 法により検出し得た日本人同遺伝子変異の特徴を解析した。

B. 研究方法

Dysferlin 遺伝子解析は、ゲノム DNA を55個のエクソンごとに近傍のイントロンを含め PCR-SSCP 法にて遺伝子変異をスクリーニングし、直接塩基配列決定法にて確認した。c.1566C>G 変異は PCR 産物の *Mbo I* による切断を見て直接塩基配列を確認した。ナンセンス変異以外は正常 100 染色体に存在しないことを確認した。

（倫理面への配慮）

遺伝子解析はインフォームドコンセントを行い、書面で同意を得て、検体を匿名化し行った。東北大学および共同研究施設である国立病院機構西多賀病院の倫理委員会で承諾を受けた。

C. 研究結果

91 家系に 42 種類の変異を見出した。その中で遺伝子変異が確定した 51 人が三好型遠位型ミオパチー、40 人が肢帯型筋ジストロフィー2B 型、1 人が distal anterior compartment myopathy、7 人が高 CK 血症の表現型を呈していた。54 家系がホモ接合、31 家系が複合ヘテロ接合だった。6 家系の発端者では 1 アレルしか変異を見出せなかった。変異は遺伝子全体に広く分布し、22 種類のナンセンス変異、10 種類のミスセンス変異、10 種類のスプライス部位の変異

だった。c.2997G>T（p.W999C）変異が40アレル(22.7%)、c.1566C>G(p.Y522X)変異が23アレル（13.1%)、c.4497delT変異が15アレル（8.5%)、c.3373delG変異が14アレル（8.0%）多い変異だった。c.3373delG変異は肢帯型筋ジストロフィー2B型には1アレルしか認めなかった。一方c.2997G>T変異は三好型遠位型ミオパチーより肢帯型筋ジストロフィー2B型に多く見られ、ホモ接合は三好型遠位型ミオパチーでは1人のみだった。

D. 考察

日本人のdysferlin遺伝子変異の特徴は遺伝子全体に広く分布し、4種類の多い変異でアレル単位では過半数をしめる。この変異は三好型遠位型ミオパチーに多く見られる。その一方でほとんど見られない遺伝子型も存在し、遺伝子型と表現型の関連が示唆される。遠位型ミオパチーの遺伝子型の効率的な確認方法はdysferlin遺伝子以外の原因遺伝子も含めて検討する必要がある。

E. 結論

三好型を中心として他の遠位型ミオパチーの実態に関する解析を行った。本研究は将来dysferlinopathyの治療研究がおこなわれる際の重要な基礎資料となると考える。

F. 研究発表

1. 論文発表

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G. 知的所有権の取得状況

1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他
   なし

厚生労働科学研究費補助金（障害者対策総合研究事業（神経・筋疾患分野））
分担研究報告書

縁取り空胞を伴う遠位型ミオパチーの治療効果最大化のための研究

～GNEミオパチー(縁取り空胞を伴う遠位型ミオパチー:DMRV)の臨床評価指標についての研究～

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研究要旨
1. 縁取り空胞を伴う遠位型ミオパチー（DMRV）患者27人の自然歴研究を行い、進行に有用な指標を考察した。

A. 研究目的

GNEミオパチー（縁取り空胞を伴う遠位型ミオパチー（DMRV））は本邦に多く集積するミオパチーであるが、*GNE*遺伝子変異が確定された患者の自然歴に関する統括的な研究がなされておらず、評価指標は明らかでない。迫り来る治験に有用な項目選定のための横断・前向き観察研究を行った。

B. 研究方法

インフォームドコンセントの得られたNCNP病院神経内科通院中の成人患者を対象にした。17筋の徒手筋力テスト（合計MMT）、6分間歩行（6MWT）、大腿四頭筋筋力測定（hand held dynamometry, HHD）、粗大運動評価尺度（Gross motor function measurement, GMFM）、握力、ピンチ力、咬合力、lean body mass、skeletal muscle mass index (SMI)、CK、%FVC、心機能(EF, FS)、心電図およびホルター心電図、Barthel Index (BI)、modified Rankin Scale (mRS)、SF-36を用いた。歩行不能群を進行群と仮定した。

（倫理面への配慮）

取り扱う情報は、遺伝子解析の結果を含む個人情報であり個人情報管理については十分な配慮を行った。本研究は厚生労働省・文部科学省「疫学研究に関する倫理指針」を準拠し、行政機関の保有する個人情報の保護に関する法律を遵守した。

C. 研究結果

27名、43.9±13.1歳（23-68）、男性 9人、女性 18人が参加した。初発症状出現は28.0±11.9（14-56）歳、独歩（6MWT）可能9名、介助歩行可能3名、歩行不能15名であった。心筋障害・不整脈は認めなかった。歩行不能者ではMMT、GMFM、HHD、握力、%FVC、lean body mass（%BW, leg）、SMI、CK、BI、mRS、SF-36のPCSとMCS が有意に低かった。一年間の前向き研究には19名が参加し、6MWT (350.2±118.2→ 283.8±140.6m、p=0.036)、GMFM (42.7±38.0→ 40.1±37.7N, p=0.071)、握力(7.0±6.7→ 5.6±5.9kg, p=0.073)、合計

MMT(17.9±9.9→ 16.1±9.9, p=0.016)が有意に低下した。合計MMT、GMFM低下は歩行不能群より可能群で大きい一方、歩行可能群で握力と6MWTの低下が見られた。

D. 考察

歩行の可否で進行する指標が異なることについては、病期により障害される部位が異なる可能性があり、評価を行う上では歩行可能者と歩行不能者は項目を分けて行う必要がある。一年間の変化はALSなどの検討と比べて緩徐であり、治験薬の治療効果判定などでは薬剤の運動能力維持効果を判定するためには前観察期間をおくことが必要と考えられる。

E. 結論

短期間で変化が見られる 6MWT、GMFM、握力、MMT は治験の評価項目の候補と考えた。歩行不能群で低下が見られる SMI、lean body mass、 BI、mRS、SF-36 も評価指標として有用な可能性がある。歩行可能者と歩行不能者では病状進行の部位が異なると推察した。GNE ミオパチーでは心筋障害は認めなかった。

F. 研究発表

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G. 知的所有権の取得状況

1. 特許取得
   なし

2. 実用新案登録
   なし

3. その他
   なし

III　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

発表者氏名： 論文タイトル名. 発表誌名 巻号： ページ, 出版年
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IV　研究成果の刊行物・別刷

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Neuromuscular Disorders 23 (2013) 84–88

www.elsevier.com/locate/nmd

Respiratory dysfunction in patients severely affected by GNE myopathy (distal myopathy with rimmed vacuoles)

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Abstract

GNE myopathy is a rare and mildly progressive autosomal recessive myopathy caused by *GNE* mutations. Respiratory dysfunction has not been reported in GNE myopathy patients. In this study, we retrospectively reviewed the respiratory function of 39 severely affected GNE myopathy patients (13 men, 26 women) from medical records, and compared these parameters with various other patient characteristics (e.g., *GNE* mutations, age at onset, creatine kinase levels, and being wheelchair-bound) for correlations. The mean % forced vital capacity [FVC] was 92 (26) (range, 16–128). In 12/39 (31%) patients, %FVC was <80%. Of these 12 patients, 11 (92%) were entirely wheelchair-dependent. These patients exhibited significantly earlier onset (20 [4] vs. 30 [8] years, $p < 0.001$) and lower creatine kinase levels (56 [71] vs. 279 [185] IU/L) than patients with normal respiratory function. Two patients exhibited severe respiratory failure and required non-invasive positive pressure ventilation. Patients with a homozygous mutation in the *N*-acetylmannosamine kinase domain exhibited lower %FVC, while only one compound heterozygous patient with separate mutations in the uridinediphosphate-*N*-acetylglucosamine 2-epimerase and the *N*-acetylmannosamine kinase domains had respiratory dysfunction. Our results collectively suggest that GNE myopathy can cause severe respiratory failure. Respiratory dysfunction should be carefully monitored in patients with advanced GNE myopathy characterized by early onset and homozygous homozygous mutations in the *N*-acetylmannosamine kinase domain.

Keywords: GNE myopathy; Distal myopathy with rimmed vacuoles (DMRV); Hereditary inclusion body myopathy; Respiratory dysfunction; Uridinediphosphate-*N*-acetylglucosamine (UDP-GlcNAc) 2-epimerase domain; *N*-acetylmannosamine kinase domain

1. Introduction

GNE myopathy, also known as distal myopathy with rimmed vacuoles (DMRV), Nonaka myopathy, or hereditary inclusion body myopathy (hIBM), is an early adult-onset, slowly progressive myopathy that preferentially affects the tibialis anterior muscle but relatively spares the quadriceps femoris muscles [1,2]. Respiratory dysfunction has not been reported in GNE myopathy [3]. Nonaka et al. reported that respiratory muscles were rarely involved even in bed-ridden patients, but no data were presented [1]. However, we had noticed that a few patients with GNE myopathy exhibited mild but progressive respiratory loss, with some experiencing recurrent pneumonia due to reduced airway clearance. Recent recommendations suggest training patients with neuromuscular disease with respiratory dysfunction using the air stacking technique to increase their thorax capacity and assisted cough peak flow (CPF) from an early stage to maintain lung compliance and chest mobility, and to clean the airways [4]. If respiratory dysfunction is not rare in patients with GNE

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myopathy, then, physicians should punctually monitor their respiratory function with pulmonary function tests to look for early signs of respiratory dysfunction, perform respiratory training, coup with airway infection using a mechanical in-exsufflator (MI-E), and induce mechanical ventilation if required, as they do for patients with neuromuscular disease who exhibit respiratory failure.

The aim of this study is to evaluate past and present clinical respiratory function test parameters of GNE myopathy patients, and analyze factors that correlate with disease severity.

2. Patients and methods

2.1. Study population

Medical records of all genetically confirmed GNE myopathy patients who underwent pulmonary function tests at the National Center Hospital, National Center of Neurology and Psychiatry, were retrospectively reviewed. We collected data on genetic diagnosis, respiratory function (% vital capacity [%VC], % force vital capacity [FVC], cough peak flow [CPF]), creatine kinase (CK), chest X-ray and/or CT scan and body mass index (BMI) for analysis.

2.2. Data handling and analysis

Data were summarized using descriptive statistics, and each variable was compared against age, sex, respiratory dysfunction (whether their %FVC was up to or over 80%), and domain mutation (i.e., within the UDP-GlcNAc 2-epimerase domain: ED or *N*-acetylmannosamine kinase domain: KD). The *t*-test was used to compare the means of each group. Data for the two study populations were calculated using chi-square contingency table analysis. Multivariate regression analysis was performed with %FVC as the dependent variable. Explanatory variables included age at disease onset, CK and BMI. We found that the variables age, duration from onset to present, age upon wheelchair use, age at loss of ambulation, were highly correlated (over 0.5) with age at disease onset. As such, we eliminated these three due to multicolinearity in the multivariate regression analysis. When past %FVC data were available, the present data were compared with serial changes in respiratory function during the preceding 5–7 years, and changes in %FVC over time were determined by calculating the difference between past and present data. All analyses were performed using SPSS for Macintosh (Version 18; SPSS Inc., Chicago, IL).

3. Results

3.1. General characteristics

A total of 39 Japanese patients (13 men, 26 women) were recruited. The mean age at the time of data collection was 43.1 (11.3) years (mean [standard deviation, SD]) (Table 1). The mean age at first appearance of symptoms was 26.8 (9.0) years (range, 15–58 years; median, 25 years). Present age, age at disease onset, age at wheelchair use, and present ambulation status were not significantly different between men and women; 20.5% (8/39) had symptom onset before age 20. Of the 39 patients, 51.3% (20/39) could walk but needed assistance, and 69.2% (27/39) were wheelchair-bound (8/27 and 19/27 were partially and totally wheelchair-bound, respectively). Age at first use of a wheelchair was 33.3 (10.8) years (range, 18–59 years; median, 31.5 years) and that for loss of ambulation was 36.9 (11.9) years (Table 1).

3.2. GNE mutations

Of the 39 patients, 30.7% (12/39) carried homozygous mutations, while 69.2% (27/39) harbored compound heterozygous mutations (Supplementary Table 1). Among the homozygous patients, 66.7% (8/12) harbored the p.V572L mutation. Among the compound heterozygous patients, 25.9% (7/27) exhibited the p.D176V/p.V572L genotype, while the other patients each had a different mutation. With respect to the location of the mutation (i.e., protein domain), 28.2% (11/39) homozygous patients carried mutations only in ED (ED/ED), 46.2% patients (18/39) were compound heterozygotes with 1 mutation each in the ED and KD (ED/KD), and 25.6% patients (10/39) had a mutation in the KD of both genes (KD/KD) (Table 2). The allelic frequencies of p.V572L, p.D165V, p.C13S, and p.R129Q were 35.9% (28/78), 28.2% (22/78), 11.5% (9/78), and 2.6% (2/78), respectively, while all other mutations had only 1 allele each (Supplementary Table 1).

3.3. Respiratory function

None of the patients had lung and/or thoracic diseases that could affect their respiratory function in chest X-ray and/or chest computed tomography. The %VC and %FVC in patients with GNE myopathy were 91.9 (26.9) (range, 18.2–126.3; median, 100.3) and 92.0 (25.8) (range, 16.4–128.5; median, 100.5; Table 1), respectively.

3.4. Patients with respiratory dysfunction

In 30.7% of patients (12/39), %FVC was <80. Of these 12 patients, 91.6% (11/12) were wheelchair-dependent and 83.3% (10/12) had already lost ambulation. Their onset was significantly earlier (19.3 [4.4] vs. 30.3 [8.4], $p < 0.001$) and mean CK level was significantly lower (55.8 [71.6] vs. 279.0 [184.7], $p = 0.004$) than those of patients with normal respiratory function. Four patients exhibited advanced respiratory dysfunction (%FVC < 50% and cough peak flow [CPF] < 160 L/min) (Table 2). All 4 patients had experienced recurrent pneumonia, and 2 patients required nocturnal NPPV. They were all early onset (before 20 years old) and non-ambu-

Table 1
Patient characteristics by respiratory function.

	Total	%FVC ⩾ 80%	%FVC < 80%	
n	*39*	27	12	*p*
Age (years)	43.0 ± 11.3	44.3 ± 11.7	39.9 ± 10.3	0.267
Age at onset (years)	26.8 ± 9.0	30.2 ± 8.4	19.2 ± 4.4	<0.001
GNE/GNE	10 (25.6%)	7 (70.0%)	3 (30.0%)	0.640
GNE/MNK	18 (46.2%)	16 (88.9%)	2 (11.1%)	0.018
MNK/MNK	11 (28.2%)	4 (36.4%)	7 (63.6%)	0.009
Duration from onset of disease to present	16.2 ± 8.4	14.1 ± 7.8	20.8 ± 8.2	0.021
Wheelchair use (%)	27 (69.2%)	16 (59.3%)	11 (40.7%)	0.141
Wheelchair use since (years)	33.3 ± 10.8	37.9 ± 11.3	26.6 ± 5.1	0.002
Lost ambulation	19 (48.7%)	8 (42.1%)	11 (57.9%)	0.014
Age at lost ambulation (years)	36.9 ± 11.9	41.2 ± 11.7	28.2 ± 6.4	0.018
CK (IU/L)	201.3 ± 187.5	279.0 ± 184.7	55.8 ± 71.6	0.004
BMI	21.1 ± 4.2	20.8 ± 3.2	21.9 ± 5.8	0.457
FVC (%)	91.9 ± 26.9	106.9 ± 12.5	58.2 ± 18.7	<0.001
VC (%)	92.0 ± 25.8	106.4 ± 11.6	59.5 ± 17.6	<0.001
CPF (L/min)	334.2 ± 139.5	378.0 ± 105.7	250.2 ± 161.5	0.008

Most patients with reduced respiratory function had already lost ambulation and were entirely wheelchair-dependent. Their onset was significantly earlier and CK levels significantly lower than those of patients with normal respiratory function. FVC: forced vital capacity, VC: vital capacity, CPF: cough peak flow, BMI: body mass index, CK: creatine kinase.

Table 2
Patients with FVC < 50% and CPF < 160 L/min.

Case	Age	Sex	Mutation	Mutant domain	Ambulation status	Disease onset	Disease duration	Age at lost ambulation	%VC	%FVC	CPF (L/min)	Reccurent pneumonea	NPPV	CK (IU/L)	BMI
1	51	Man	p.C13S homozygote	ED/ED	Non-ambulant	17	34	25	18.2	16.4	48.0	Yes	Nocturnal	13	18.6
2	42	Woman	p.V572L homozygote	KD/KD	Non-ambulant	16	26	23	37.6	34.4	141.6	Yes	Nocturnal	13	22.2
3	45	Woman	p.V572L homozygote	KD/KD	Non-ambulant	17	28	31	49.0	48.3	147.6	Yes	No	8	31.6
4	37	Woman	p.V572L homozygote	KD/KD	Non-ambulant	16	21	24	53.7	48.6	118.8	Yes	No	No data	20.4

Table 3
Multivariate regression analysis of predictive factors for respiratory dysfunction.

	Regression coefficient	*p*	Lower limit of 95% confidence interval	Upper limit of 95% CI
Age at onset	0.949	0.042	0.038	1.86
CK	0.068	0.008	0.02	0.115
BMI	−1.8	0.09	−3.811	0.302

Multivariate linear regression analysis was performed to evaluate the relationship between %FVC and other clinical parameters. Age at onset and CK were significantly correlated with %FVC.

lant. The majority (7/12) of patients had KD/KD mutations, whereas significantly fewer patients with respiratory dysfunction had ED/KD mutations.

In order to identify predictive factors for respiratory dysfunction in GNE myopathy, we performed multivariate analysis to determine the relationship with %FVC. This revealed age at onset ($p = 0.042$) and CK ($p = 0.008$) as significantly correlated to %FVC (Table 3, Fig. 1).

Past (5–7 years ago) data were available for 9 patients. The %FVC decrements in 5 patients with respiratory dysfunction were significantly greater than those of patients without dysfunction (20.9 [6.0] vs. 0.8 [9.7], $p = 0.004$; Supplementary Table 2).

4. Discussion

To our knowledge, we are the first to report respiratory dysfunction in GNE myopathy. Our study demonstrates that (1) certain GNE myopathy patients in Japan exhibit respiratory dysfunction, and (2) early onset and lower CK levels resulting from severe muscle atrophy and weakness, and KD/KD mutations can be risk factors for respiratory dysfunction.

Malicdan et al. reported that pathological changes in the diaphragms of the GNE (−/−) hGNED176V-Tg model mice were variable and ranged from almost normal to the presence of marked fibrosis and rimmed vacuoles. On the other hand, the gastrocnemius muscles of all mice exhibited myopathic features [5]. The features in these mice correspond to individual differences observed in the patients of our study. The fact that not all cases in our study exhibited respiratory dysfunction as observed in the GNE (−/−)

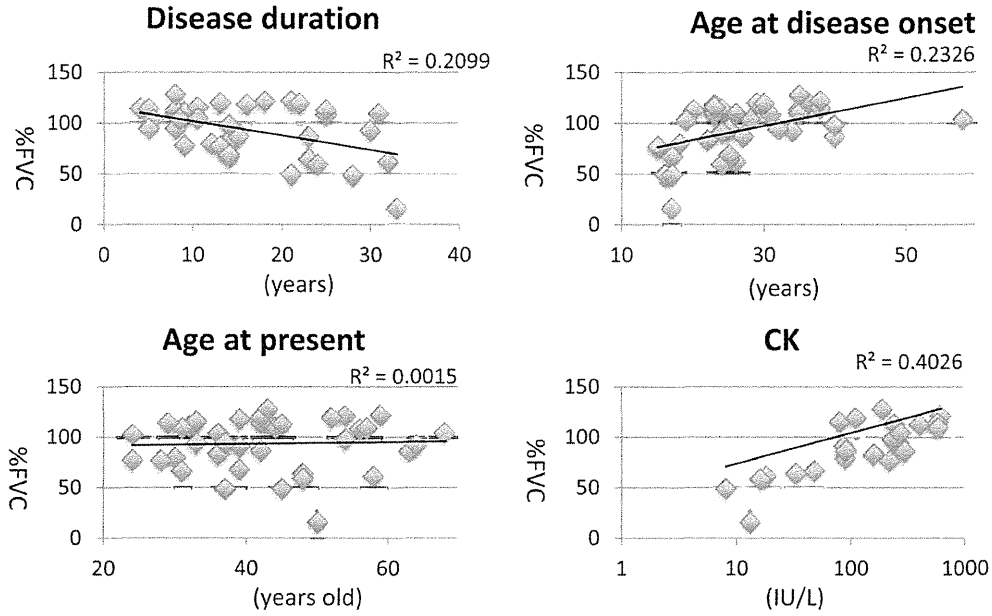

Fig. 1. Scatterplots of %FVC as functions of age, age at disease onset, disease duration, and creatine kinase (CK) level. Age at disease onset, disease duration, and CK level were correlated with %FVC.

hGNED176V-Tg mice indicates that severe respiratory muscle involvement is not a constant feature of GNE myopathy. Yet, since about 30% of patients had decreased %FVC and severe respiratory dysfunction was overlooked by neurologists or physicians, clinicians should be made more aware of the possibility of respiratory dysfunction, particularly in patients with advanced GNE myopathy. If %VC decreases to 70%, patients should be taught air stacking as with other neuromuscular disorders [4,6]. CPF should be routinely measured in patients with GNE myopathy, given that its decrement was associated with recurrent pneumonia in our study. Early induction of assisted CPF and/or MI-E is required if patients with reduced CPF have an airway infection. Serial data suggest that %FVC decreased from the normal range to %FVC < 80, indicating that continuous monitoring is required even in patients with normal respiratory function. Moreover, respiratory function parameters may provide quantitatively useful data for clinical trials, particularly those directed to non-ambulant patients.

All 4 patients with severe respiratory dysfunction exhibited early onset, homozygous mutations, and advanced muscle weakness. However, not all early onset, homozygous, or non-ambulant patients exhibited severe respiratory dysfunction. Although the underlying reasons are unclear, we also found that ED/KD mutations were less associated with decreased respiratory function, while many patients with KD/KD mutations showed respiratory dysfunction. A large scale, cross-sectional study could better identify key factors responsible for respiratory dysfunction and genotype-phenotype correlations.

We are aware that the recruitment of patients from NCNP, highly specialized for muscle disease, is a potential source of selection bias, because they may be particularly more severely affected than the general patient population. Therefore, our study may not correctly reflect the general patient population. Investigations of small populations may underestimate the statistical significance as well. However, our previous GNE myopathy questionnaire study revealed a similar correlation between genotypes and phenotypes [7]. We are currently in the process of establishing a Japanese national GNE myopathy patient registry called Registration of Muscular Dystrophy (REMUDY, http://www.remudy.jp) to perform a broader epidemic investigation of associated conditions, including respiratory dysfunction. To clarify the relationship between respiratory dysfunction and other clinical/laboratory factors, we have initiated a prospective observational study on GNE myopathy.

Three of 4 patients with severe respiratory dysfunction had homozygous p.V572L mutations. Given the frequency of the p.V572L mutation in the Japanese population, it will be interesting to determine whether non-Japanese individuals harboring this mutation also exhibit respiratory dysfunction.

In conclusion, advanced GNE myopathy patients are at risk for respiratory dysfunction. The KD/KD genotype, early onset, loss of ambulation/wheelchair use, and low CK level resulted in advanced muscle atrophy may be associated with respiratory dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nmd.2012.09.007.

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Rimmed Vacuoles in Becker Muscular Dystrophy Have Similar Features with Inclusion Myopathies

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Abstract

Rimmed vacuoles in myofibers are thought to be due to the accumulation of autophagic vacuoles, and can be characteristic in certain myopathies with protein inclusions in myofibers. In this study, we performed a detailed clinical, molecular, and pathological characterization of Becker muscular dystrophy patients who have rimmed vacuoles in muscles. Among 65 Becker muscular dystrophy patients, we identified 12 patients who have rimmed vacuoles and 11 patients who have deletions in exons 45–48 in *DMD* gene. All patients having rimmed vacuoles showed milder clinical features compared to those without rimmed vacuoles. Interestingly, the rimmed vacuoles in Becker muscular dystrophy muscles seem to represent autophagic vacuoles and are also associated with polyubiquitinated protein aggregates. These findings support the notion that rimmed vacuoles can appear in Becker muscular dystrophy, and may be related to the chronic changes in muscle pathology induced by certain mutations in the *DMD* gene.

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Introduction

Rimmed vacuoles (RVs) can be seen in a certain range of muscle diseases including distal myopathy with rimmed vacuoles (DMRV) and sporadic inclusion body myositis (sIBM), myofibrillar myopathies, and also lysosomal myopathies [1–3]. By lysosomal enzymatic activities and electron microscopic features, RVs represent accumulation of autophagic vacuoles [4]. RVs are thought to be due to lysosomal dysfunction or due to accumulation of the various proteins that affect progression of the autophagic process within myofibers [1–2,5].

Becker muscular dystrophy (BMD) is a dystrophinopathy caused by mutations in *DMD* gene that shows a milder disease course as compared to Duchenne muscular dystrophy (DMD). BMD patients show a wide variety of symptoms from gait disturbance in early childhood to almost no weakness even in adulthood. Through our muscle repository, we noted that some dystrophinopathies also show RVs in the muscles, albeit rare [6]. Because dystrophinopathies are related to membrane fragility of myofibers, the presence of RVs in BMD patients is perplexing and raises several issues that need to be clarified, like the relevance of RVs in BMD and the frequency of BMD patients that show RVs in myofibers. The second issue is the clinical and pathological features of BMD muscles associated with RV formation. The third issue is the characters of the RVs in BMD in comparison with those seen in the other disorders.

In this study, we focused on BMD patients who showed RV in muscle biopsy sections, and noted genetic and clinical characteristics, in addition to features seen in muscle pathology. Extensive immunohistochemical analysis was performed to note the nature of these RVs in comparison to those seen in IBM.

Materials and Methods

Ethics Statement

This study was approved by the ethics committee in National Center for Neurology and Psychiatry, Japan. All data from patients were obtained through written informed consent.

Patients

From the muscle repository of National Center for Neurology and Psychiatry, we identified patients having deletion and mutation in *DMD* gene. The clinical information of each patient was carefully reviewed, and the following data were included for analysis: age at onset of disease, age at biopsy, disease duration, and serum creatine kinase (CK) level. For control, we included samples from patients genetically diagnosed as DMRV ($n = 2$) or sIBM ($n = 2$).

Histochemistry

All biopsied muscles were frozen in liquid nitrogen-cooled isopentane and kept at −80°C. Transverse serial sections of frozen muscles with 8 μm thickness were stained with H&E, modified Gomori trichrome (mGT) and a battery of histochemical methods, including acid phosphatase and nonspecific esterase [7].

Table 1. Summary of clinical and pathological findings of BMD patients with rimmed vacuoles.

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12
DMD exon deletion/point mutation	45–47	45–47	45–47	45–47	45–48	45–48	45–48	45–48	45–48	45–53	14–41	c.5827A>G p.Met1943Val
Clinical findings												
age at onset (years)	6	13	20	20	5	13	35	44	33	39	32	57
symptom at onset	cramp	weakness	weakness	weakness	hypertrophy	weakness	weakness	pain	weakness	weakness	weakness	weakness
age at biopsy (years)	34	35	41	43	22	41	49	54	74	45	34	60
serum CK activity (IU/L)	1233	3061	1076	1136	2788	981	481	1327	2420	830	2428	2424
Pathological findings												
biopsied muscle	BB	unknown	BB	BB	unknown	BB	QF	RF	BB	QF	QF	BF
fiber type population												
type 1 (%)	10	40	40	82	37	36	7	21	76	41	12	84
type 2A (%)	38	16	33	12	23	23	36	41	13	32	30	11
type 2B (%)	50	42	23	1	38	41	55	36	5	24	10	1
type 2C (%)	2	2	4	5	2	1	2	2	6	3	1	4
fibers with internally placed nuclei (%)	10	15	30	5	25	50	50	15	10	30	15	70
fibers with rimmed vacuole*	10	42	5	2	6	21	29	7	11	78	1	13
small atrophic fibers*	10	154	137	57	4	42	88	7	11	354	0	26
necrotic fibers*	0	0	5	0	0	2	0	3	4	3	2	0
regenerating fibers*	2	2	32	0	12	2	0	7	4	3	2	9

BB = biceps brachii; QF = quadriceps femoris; RF = rectus femoris; BF = biceps femoris; *per 1,000 fibers

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Table 2. Summary of clinical and pathological findings of BMD patients without RVs (*DMD* deletion exons 45–47 and 45–48).

Patient No.	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
DMD Deletion	**exons 45–47**									**exons 45–48**									
Clinical findings																			
age at onset (years)	22	30	45	14	4	unknown	38	14	5	4	5	13	15	16	2	45	7	33	39
symptom at onset	weakness	weakness	weakness	weakness	atrophy	unknown	weakness	high CK	pain	pain	weakness	pain	weakness	weakness	cramp	weakness	weakness	weakness	weakness
age at biopsy (years)	24	46	60	48	19	23	48	14	6	14	10	53	58	64	16	48	10	56	40
serum CK activity (IU/L)	994	1487	942	569	1193	unknown	321	2705	15540	3044	11174	549	513	245	2702	758	2945	615	600
Pathological findings																			
biopsied muscle	QF	deltoid	BB	BB	QF	unknown	BB	GC	BB	BB	BB	BB, QF	BB	GC	QF	BB	BB	TA	GC
fiber type population																			
type 1 (%)	10	66	36	42	16	54	70	28	46	41	38	50	67	47	27	40	48	80	54
type 2A (%)	44	28	47	49	67	23	22	59	32	24	55	26	28	31	51	38	45	20	30
type 2B (5)	46	3	16	10	9	22	4	11	20	33	4	21	6	13	12	17	4	0	16
type 2C (%)	0	3	1	0	0	2	4	2	2	2	3	3	0	8	10	5	3	1	1
small atrophic fibers*	73	32	0	278	56	62	115	3	11	16	7	115	0	78	150	51	24	6	80
necrotic fibers*	0	0	0	0	0	8	1	3	2	3	1	0	0	2	3	0	5	0	0
regenerating fibers*	4	22	0	0	0	0	3	13	13	1	15	2	0	0	3	2	7	3	5

QF = quadriceps femoris; BB = biceps brachii; GC = gastrocnemius; TA = tibialis anterior; *per 1,000 fibers

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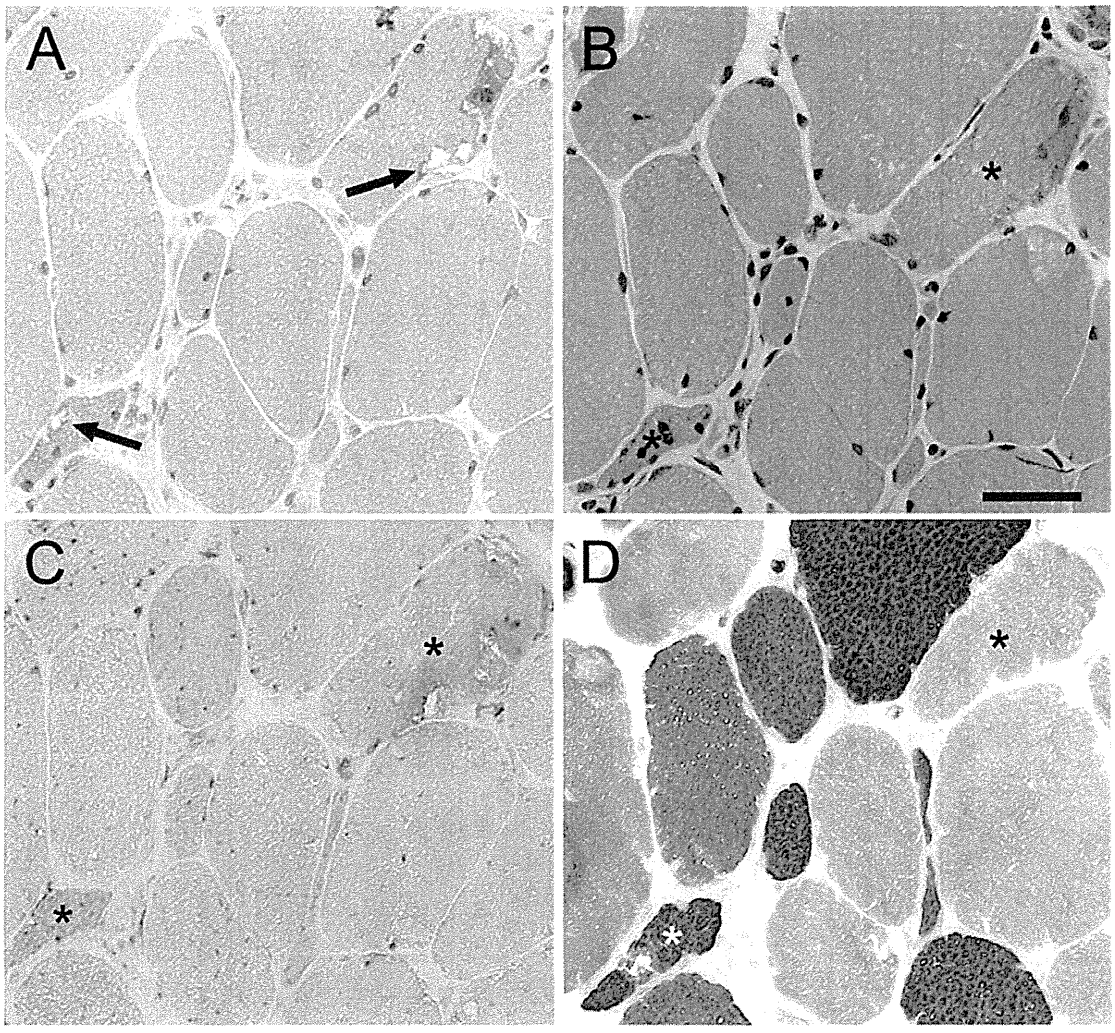

Figure 1. Pathological Characteristics of BMD patients. A: On mGT staining, RVs are seen in the periphery of myofibers (**arrow**). **B:** On H&E staining, there is marked variation in fiber size with scattered small atrophic fibers. **C:** High acid phosphatase activity is seen in the areas of RVs. **D:** On ATPase staining pre-incubated at pH 4.6, RVs are seen in both type 1 and type 2 fibers. **Asterisks** indicate myofibers with RVs. Scale bar: 25 μm.
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For histological analysis, the following parameters were noted for the evaluation of specific pathological characters: number of necrotic and regenerating fibers (defined as homogeneously pink and basophilic fibers on H&E staining, respectively); fiber type composition as determined by ATPase staining with pre-incubation at pH 4.6 and pH 10.0; occurrence of RVs seen on mGT staining; number of atrophic fibers; and other characteristic pathology. All routine histochemistry and immunohistochemistry analysis were done on adjacent serial sections. Modified gomori stain was done before and after immunohistochemistry panel to ensure the presence of rimmed vacuoles in the slides. Microscopic observation was performed using OLYMPUS BX51 (Olympus).

Genetic Analysis

Genomic DNA of patients was isolated from peripheral blood or muscle specimen using standard protocols. Multiplex ligation-dependent probe amplification (MLPA) or multiplex PCR method were done using standard protocols [8]. Genomic sequencing analysis of all the exons and flanking introns of the *DMD* gene was done in patients without deletion by MLPA. Sequence for primers used for *DMD* gene analysis are available upon request.

Immunohistochemistry

We performed indirect immunofluorescence staining on 7-μm serial sections of muscle according to previously described methods [9,10]. After immersion in a blocking solution, sections were then incubated at 37°C for 2 hours with primary antibodies against dystrophin (DYS-1, DYS-2 and DYS-3, 1:500, 1:50 and 1:10) (Novocastra), sarcoglycans (SGCA, SGCB, SGCG, and SGCD, 1:500, 1:20, 1:500 and 1:20) (Novocastra), laminin-α2 chain (1:50,000) (ALEXIS), α- and β-dystroglycan (1:50 and 1:100) (Upstate Biotech), dysferlin (1:2,500) (Novocastra), emerin (1:20) (Novocastra), collagen VI (1:2,500) (Novocastra), HLA-ABC (1:5,000) (DAKO), caveolin-3 (1:200) (Transduction laboratories), lysosomal associated membrane protein 1 (LAMP-1) (1:50) (DSHB), LC3 (1:100) (Novus biologicals), amyloid precursor protein (APP) (1:200) (Covance), beta-amyloid 1–42 (Aβ1-42) (1:100) (Chemicon), polyubiquitin (polyUb) (1:100) (Biomol),

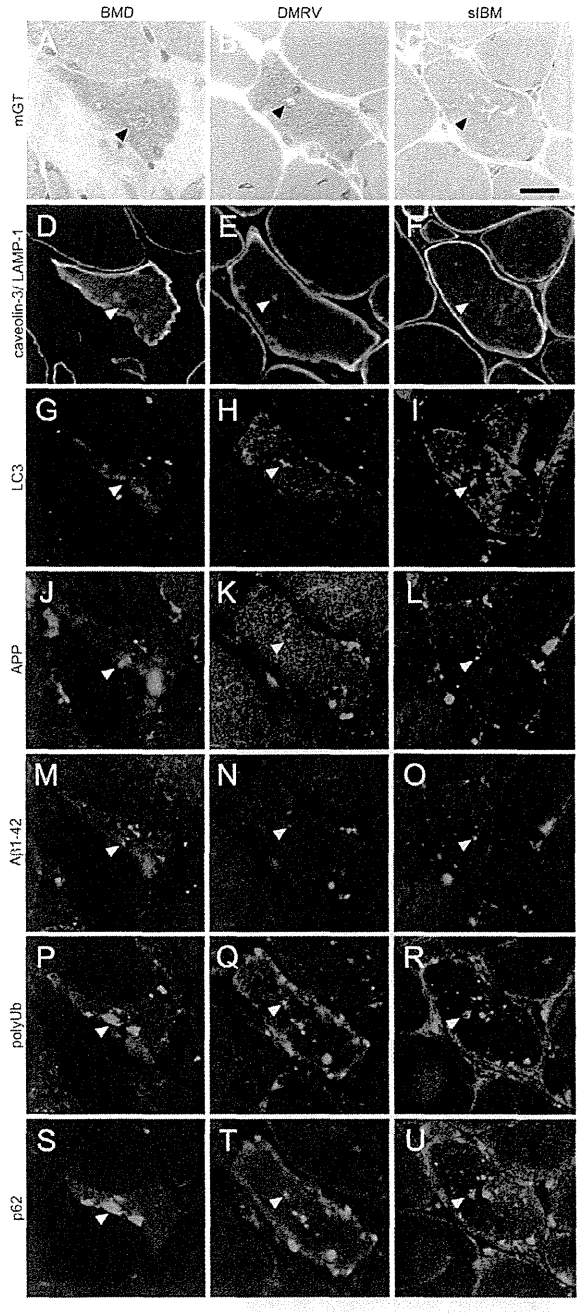

Figure 2. Immunohistochemical characteristics of RV in BMD compared to DMRV and sIBM. Representative transverse serial sections of biopsied skeletal muscles from BMD with RV (left column), DMRV (center column) and sIBM (right column) patients. **A–C:** mGT staining similarly highlights the fibers with RVs (**arrowheads**) in all patients. **D–F:** LAMP-1 (red) co-stained with caveolin-3 (green), **G–I:** LC3, **J–L:** APP, **M–O:** Aβ1-42, **P–R:** polyUb proteins, and **S–U:** p62. Immunofluorescent signals are observed around RVs (**arrowhead**). Scale bar: 25 μm.
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CD68 (KP1) (1:100) (Dako) and p62/SQSTM1 (1:200) (Biomol). After washing, slides were subsequently incubated at room temperature for 30 minutes with a secondary antibody, either Alexa-labeled donkey anti-mouse or anti-rabbit IgG (1:600) (Invitrogen), or rhodamine-labeled goat anti-mouse IgM (1:600) (TAGO), as appropriate. Sections were observed using KEYENCE BZ-9000 and digital images were analyzed by BZ-II Analyzer 1.03 (KEYENCE).

Electron Microscopy

Biopsied muscles were fixed in buffered 2% isotonic glutaraldehyde at pH 7.4, post-fixed in osmium tetroxide, dehydrated, and then embedded in Epoxy resin, according to standard protocols [7]. Ultra-thin sections were stained with uranyl acetate and lead nitrate, and observed under a Tecnai Spirit Transmission Electron Microscope (FEI).

Statistical Tests

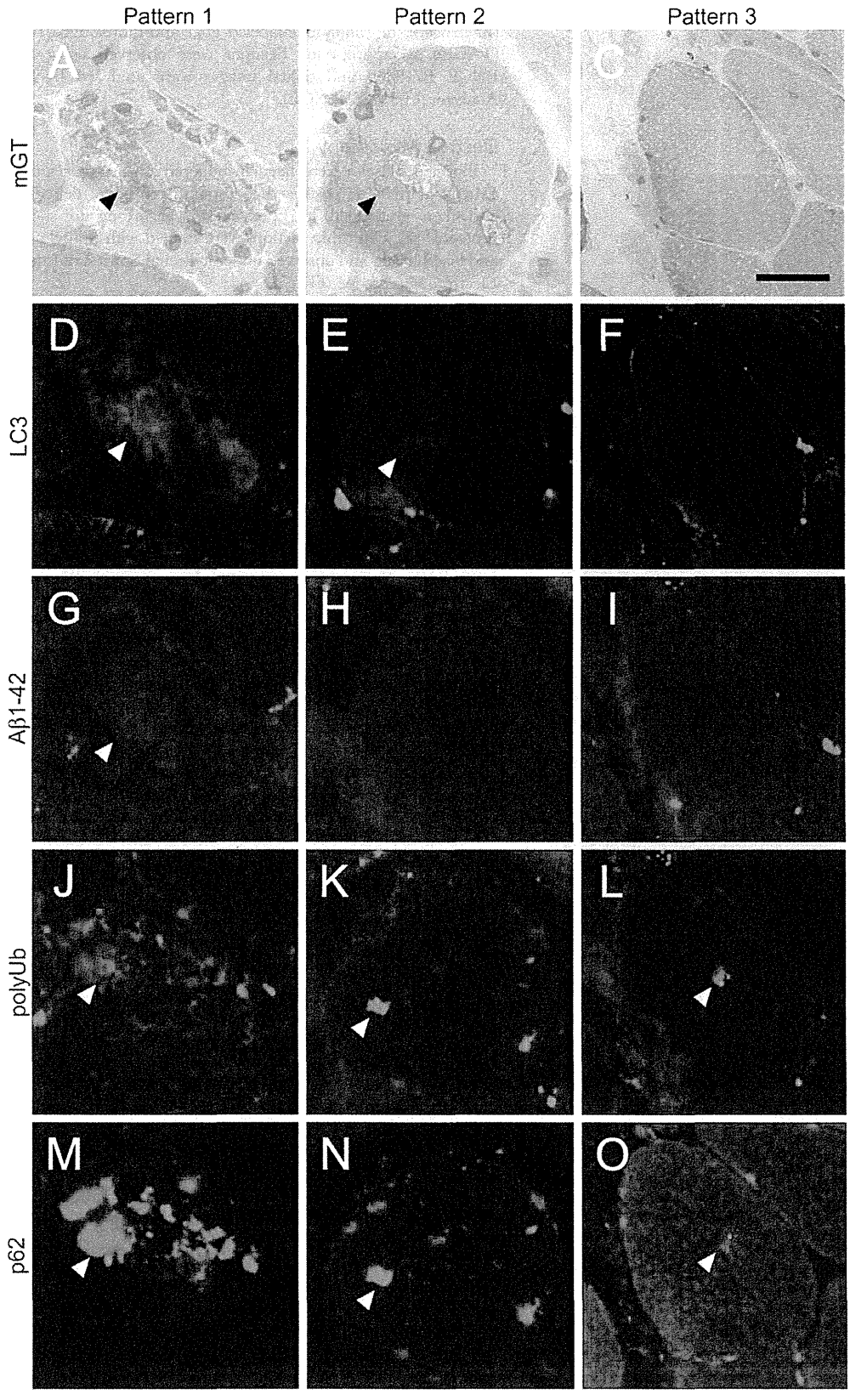
For analyzing clinical information of BMD patients with RVs as compared to BMD patients without RVs, non-parametric Mann-Whitney test or unpaired *t* test with Welch correction were used as appropriate. A *P* value less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software).

Results

Our patient cohort was composed of 65 patients diagnosed to have BMD as supported by *DMD* gene deletion (64/65) and mutation (1/65). Among these BMD patients, we identified 12 patients (18.5%) who had RVs in myofibers on muscle biopsy. By MLPA and multiplex PCR, 4 patients had in-frame deletions of exons 45–47, 5 patients had deletions of exons 45–48, one had deletion of exons 45–53, and one had deletion in exons 14–41. The remaining patient (Patient 12) was identified to be carrying a novel missense mutation (c.5827A>G, p.Met1943Val, in exon 41; Table 1) by direct sequencing of *DMD* gene. This mutation was not identified in 100 control chromosomes. We excluded the involvement of mutations in *GNE* gene, which is a causative gene for DMRV by Sanger sequencing (data not shown).

We use the term "BMD with RV" to delineate the patients who had RVs from the "BMD without RV" patients who did not have RVs in muscle sections. Deletions of exons 45–47 and 45–48 in *DMD* gene were frequent in both of BMD patient groups and the frequency of these two mutations was 9 out of 12 patients (75%) in BMD with RV group and 18 out of 53 patients (35%) in BMD without RV group. We further analyzed clinical information of only patients with deletions of exons 45–47 and 45–48 (BMD with RV patients 1–9 in Table 1 and BMD without RV group in Table 2). In terms of demographic data, BMD with RV patients were slightly older at age of disease onset (21.0±4.5 years in BMD with RV versus 19.5±3.5 years in BMD without RV, $P=0.3$), age at biopsy (43.7±4.9 years in BMD with RV versus 34.5±4.7 years in BMD without RV; $P\leq0.002$) and slightly longer mean disease duration (22.7±3 years in BMD with RV versus 15.4±3.9 years in BMD without RV; $P=0.15$). Serum CK levels in BMD with RV was slightly lower (1611±301 IU/L in BMD with RV versus 2605±964 IU/L in BMD without RV; $P=0.12$).

With regards to muscle histochemistry, all RVs in BMD were highlighted on mGT staining (Figure 1A, arrow). In addition, BMD with RV patients revealed myopathic change with moderate variation in fiber size by the presence of scattered small atrophic and angular fibers, while necrotic and regenerating fibers are rare, on H&E staining (Figure 1B). Acid phosphatase staining confirmed strong lysosomal enzyme activities within fibers with RVs (asterisks in Figure 1C). On ATPase staining, RVs were seen in both type 1 and type 2 fibers (Figure 1D) and small atrophic fibers were not


Pattern 1
Pattern 2
Pattern 3
mGT
LC3
Aβ1-42
polyUb
p62
A
B
C
D
E
F
G
H
I
J
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L
M
N
O