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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
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2. 実用新案登録
特になし

3. その他
特になし

II　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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III　研究成果の刊行物・別刷

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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 (−/−)

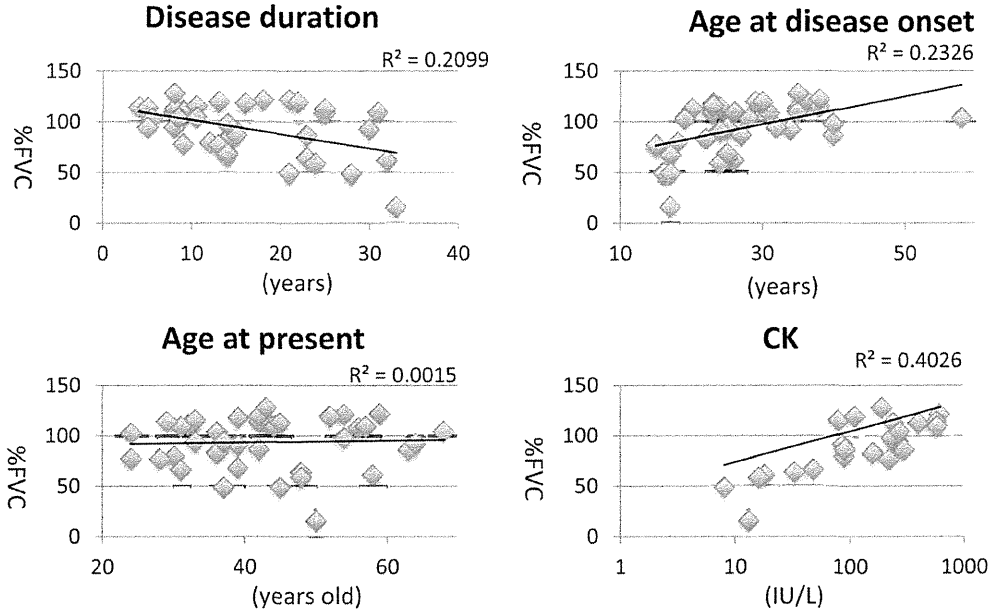

Fig. 1. Scatterplots of %FVC as functions of age, age at disease onset, disease duration, and creatine kinase (CK) level. Age at disease onset, disease duration, and CK level were correlated with %FVC.

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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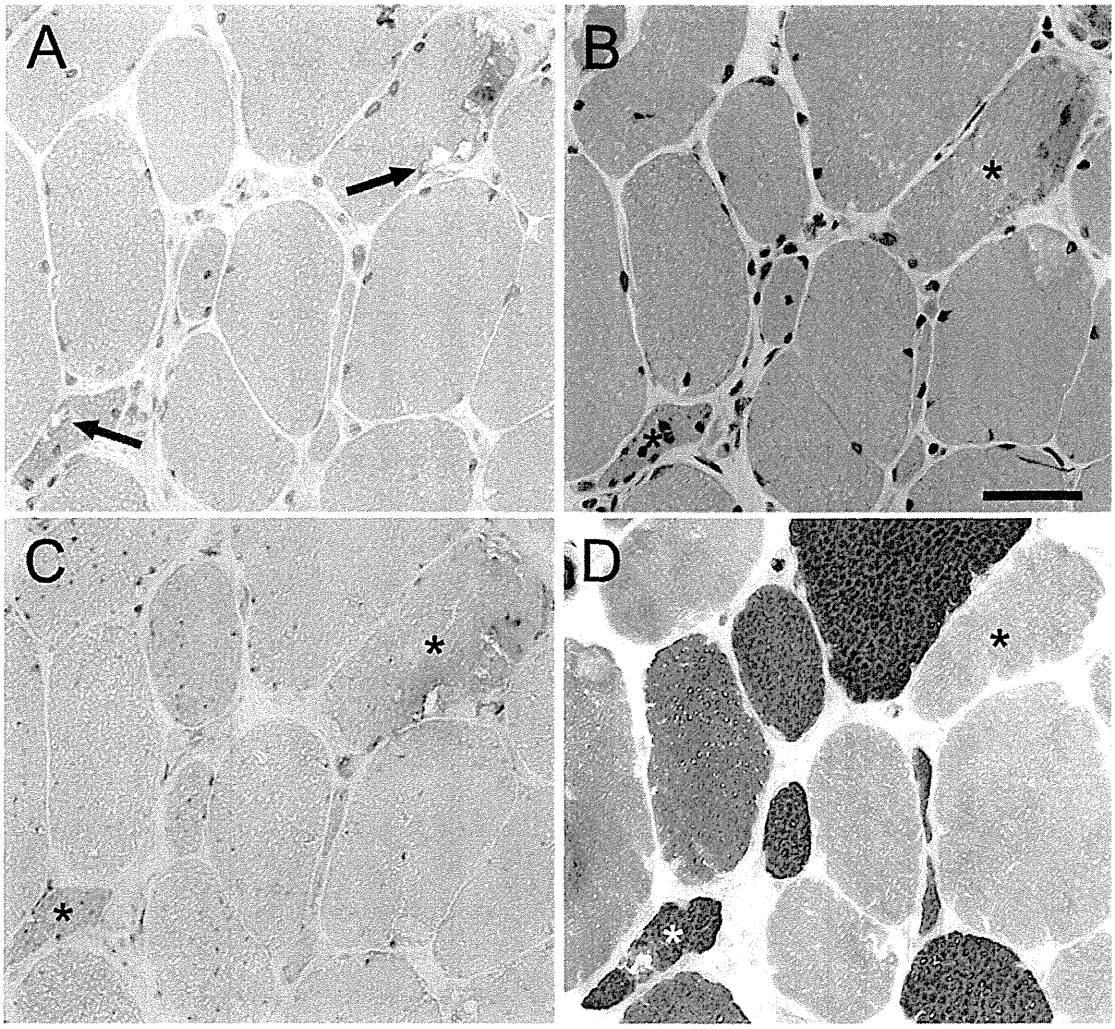


Figure 1. Pathological Characteristics of BMD patients. A: On mGT staining, RVs are seen in the periphery of myofibers (**arrow**). **B:** On H&E staining, there is marked variation in fiber size with scattered small atrophic fibers. **C:** High acid phosphatase activity is seen in the areas of RVs. **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.
doi:10.1371/journal.pone.0052002.g001

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),

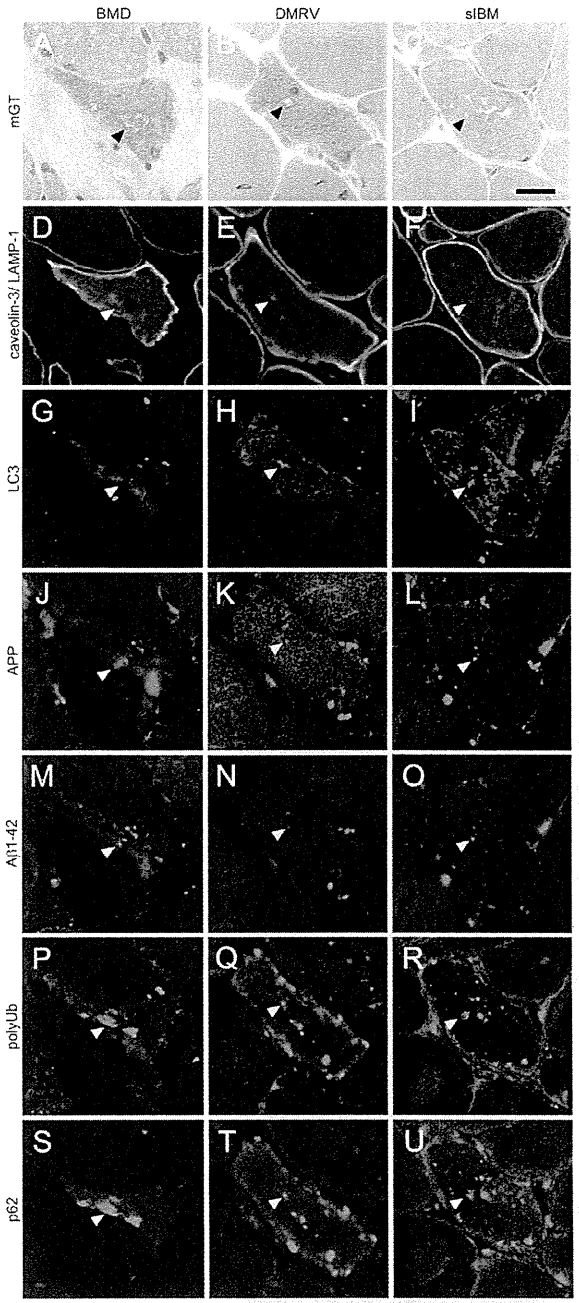


Figure 2. Immunohistochemical characteristics of RV in BMD compared to DMRV and sIBM. Representative transverse serial sections of biopsied skeletal muscles from BMD with RV (left column), DMRV (center column) and sIBM (right column) patients. **A–C:** mGT staining similarly highlights the fibers with 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.
doi:10.1371/journal.pone.0052002.g002

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

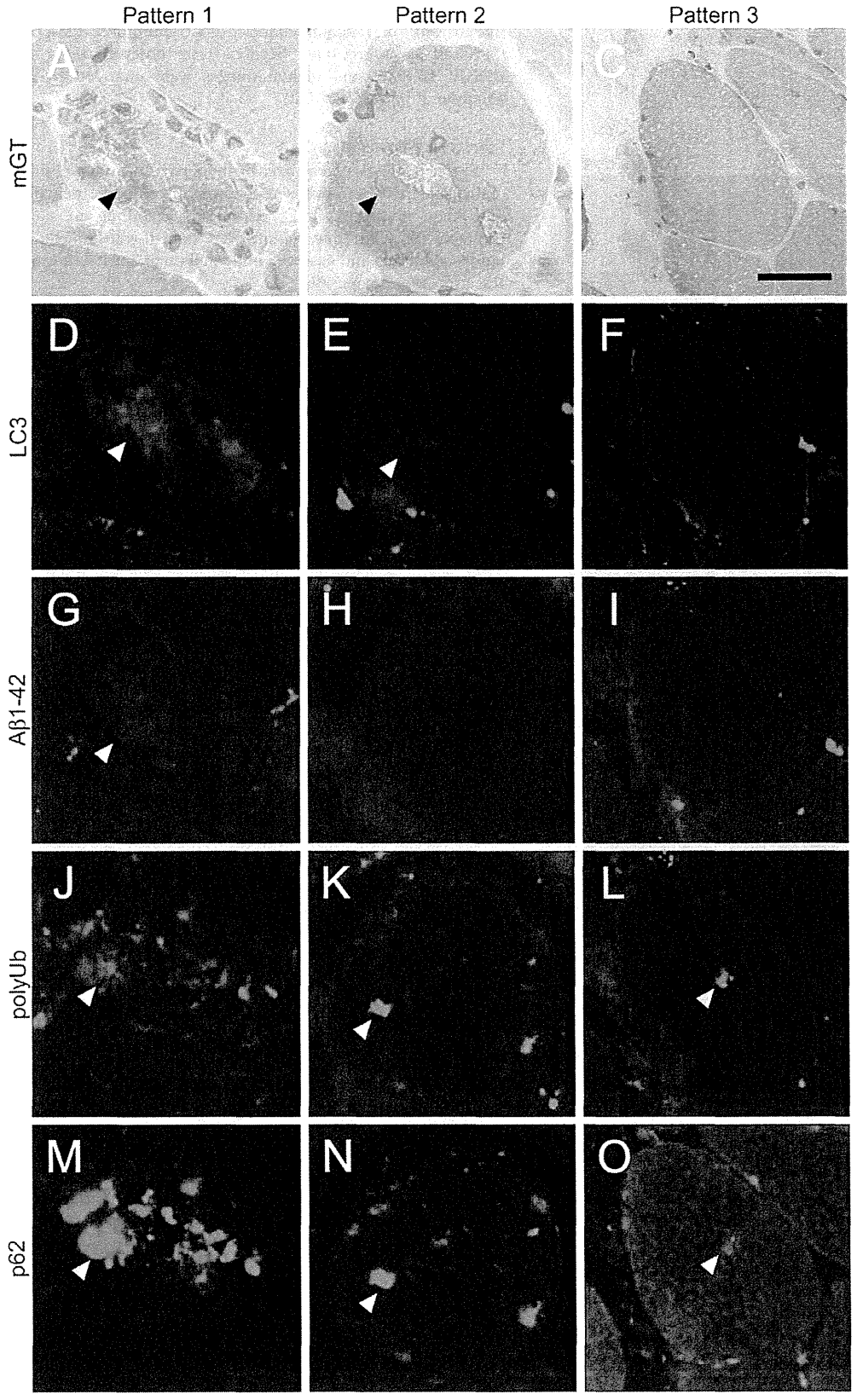
For analyzing clinical information of BMD patients with RVs as compared to BMD patients without RVs, non-parametric Mann-Whitney test or unpaired *t* test with Welch correction were used as appropriate. A P value less than 0.05 was considered significant. Statistical analysis was performed using GraphPad 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
K
L
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N
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Figure 3. Patterns of immunohistochemical findings in BMD with RV. Representative transverse serial sections of biopsied skeletal muscles from BMD patients with RV. **A–C:** mGT shows the presence of RVs (**arrowheads**). **D–F:** LC3, **G–I:** Aβ1-42, **J–L:** polyUb, and **M–O:** p62. Immunofluorescent signals are seen within the fibers with RVs (**arrowheads**). Pattern 1 (left column) shows similar characteristic staining of RV fibers as DMRV and sIBM. Pattern 2 (center column) show almost similar characteristics as pattern 1, except for the faint staining of Aβ1-42. Pattern 3 (right column), with rare occurrence, shows myofibers with RVs that are negatively stained by LC3 and Aβ1-42. Scale bar: 25 μm.
doi:10.1371/journal.pone.0052002.g003

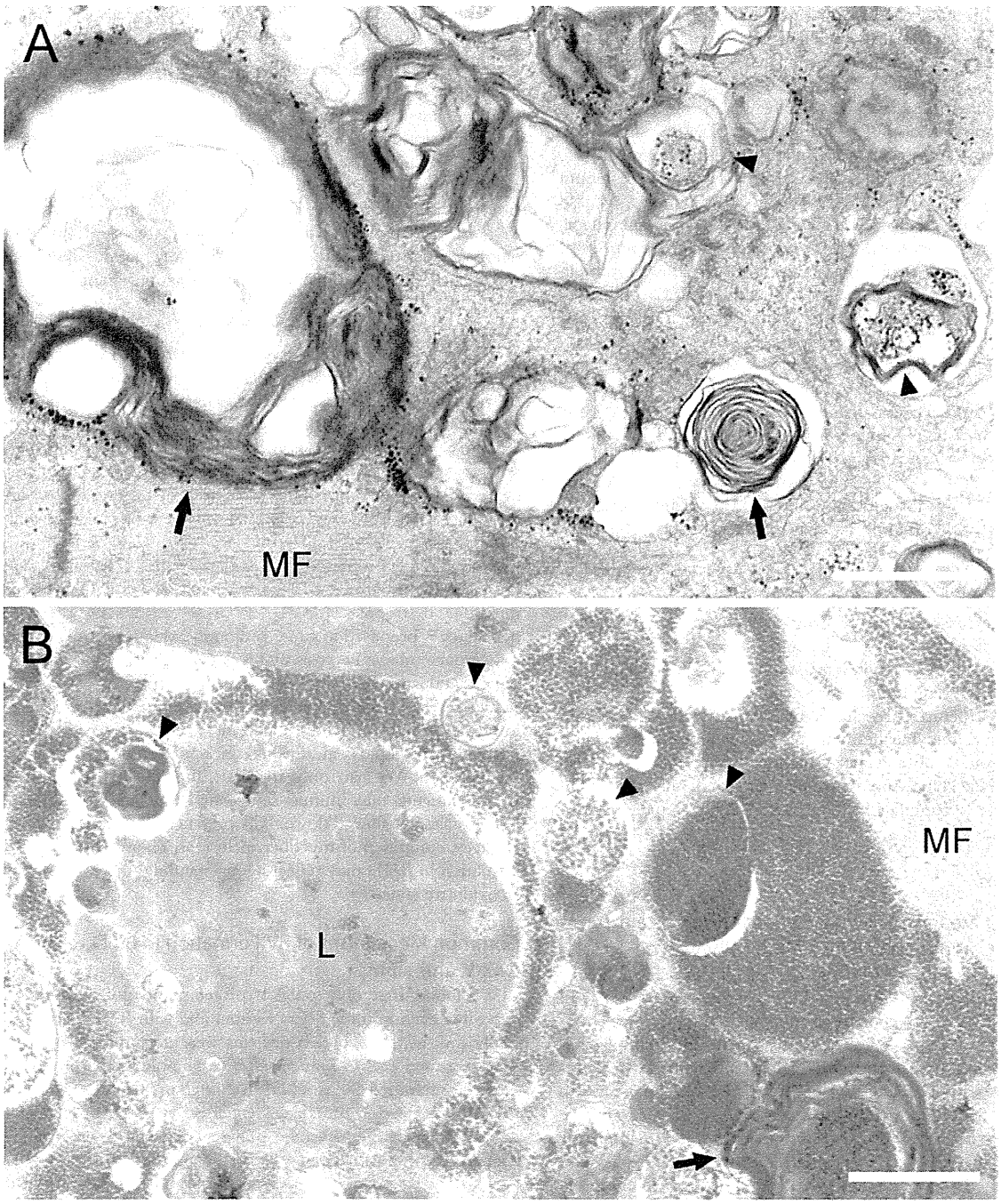

Figure 4. Areas of RVs in BMD myofibers show typical electron microscopic characteristics of autophagic vacuoles. A: Accumulation of autophagic vacuoles (**arrowheads**), various cellular debris, and multilamellar bodies (**arrow**) are seen in myofibers of some BMD patients. Note the intact arrangement of myofibrils (**MF**) surrounding autophagic area. B: In areas with or without autophagy, lipofuscin deposit (**L**) is seen. Scale bars: 1 μm.
doi:10.1371/journal.pone.0052002.g004

type 2C fibers. Positive correlation ($R^2 = 0.790$) between number of fibers with RVs and that of small atrophic fibers is seen (data not shown), suggesting a close relationship on the occurrence of RVs and the presence of atrophic myofibers.

To further characterize RVs in dystrophinopathy, we performed immunohistochemical analysis in BMD with RV patients (Figure 2, left column) in comparison with DMRV (Figure 2, center column) and sIBM (Figure 2, right column) patients; reference RVs are shown in mGT (Figure 2A–C). In RVs and areas in proximity, the lysosomal protein LAMP-1 (Figure 3D–F) and the autophagic vacuole marker LC3 (Figure 2G–I) were positively stained. As accumulation of several proteins is considered to be associated to RV formation in DMRV and sIBM [1,11,12], we observed the accumulation of APP (Figure 2J–L), Aβ1-42 (Figure 2M–O) and polyUb protein (Figure 2P–R) in and around RV in BMD with RV, DMRV and sIBM patients. We also tried to examine p62, which marks proteins for autophagic degradation in the sites with polyUb protein accumulation [13]. The staining pattern of p62 was similar to that of polyUb protein (Figure 2S–U). We also stained sections from BMD with RV, DMRV and sIBM patients with CD68 antibody, a macrophage marker, and Alexa-labeled anti-mouse IgG secondary antibody alone. Both staining were negative in RV positive fibers (Figure S1).

From our immunohistochemical analysis, we classified three patterns of staining. First, most myofibers with RV were immunoreactive to amyloid, polyUb proteins and p62 (Figure 3, left column; Pattern 1). Second, some fibers with RV showed negative for amyloid but positive for polyUb proteins and p62 (Figure 3, center column; Pattern 2). Interestingly, the third pattern consisted of some myofibers without RV that are positively stained only with polyUb proteins and p62 (Figure 3, right column; Pattern 3).

To have a closer look at the structure of these fibers with RV, we performed electron microscopy and observed the presence of autophagic vacuoles and multilamellar bodies within myofibers. In the areas surrounding autophagic vacuoles (Figure 4A), however, myofibrillar structures are almost maintained except for partial distortion of Z-line. Furthermore, lipofuscin granules were also observed around autophagic vacuoles (Figure 4B). We also confirmed Nile blue staining and confirmed that lipofuscin granules were accumulated in the fibers of BMD patients with RVs (data not shown).

Discussion

RVs in BMD are Rare but may be Related to Certain Types of *DMD* Mutations that Cause Milder Phenotype

We found 12 patients who showed RVs in muscle pathology among 65 BMD patients, representing surprising rate of 18.5%. In DMD and BMD, a genotype-phenotype correlation has been established [14–17]. Deletion of exons 45–55, for example, has been reported to be associated with quite mild muscle weakness [18–19]. Interestingly, in a previous report, one BMD patient who showed RV in his skeletal muscle section had a deletion from exons 45–48 in the *DMD* gene and showed mild to moderate weakness in lower girdle muscles [6]. In our series, the deletions in exons 45–47 or 45–48 in *DMD* gene were frequently found in the patients with RV. Our BMD with RV patients also showed a mild course of disease, with later onset and mild elevation of CK, similar as previous reports on the patients with the same deletion on *DMD* gene [6]. Additionally, in spite of similar age of disease onset in the patients with and without RV with the deletions in exons 45–47 or 45–48, the higher mean biopsy age in the patients with RV may suggest that the milder clinical course and longer disease duration in dystrophinopathy could contributory for the formation of RV in muscle. It should be noted, however, that the similar clinical course, in age of onset and biopsy, and serum CK activity, can be found in some patients in both groups, BMD with or without RV, implying that RV formation may be one of the phenotypes in patients with such deletions, or one that belongs to the disease spectrum of a mild myopathic process.

BMD Patients with RV Show Chronic Myopathic Features in Histology

Consistent with serum CK level, there were scattered necrotic and regenerating fibers observed in muscles of BMD patients without RV, while BMD patients with RV rarely show necrotic and regenerating or type 2c fibers. An increase in the number of small atrophic fibers in BMD patients with RV was remarkable as that in the patient who is previously reported [6]. This characteristic pathology is rather like myopathic changes as observed in other late-onset chronic myopathies [20].

Lipofuscin Accumulation in BMD

The number of lipofuscin granules was strikingly higher in myofibers of BMD with RV patients. Lipofuscin pigments consist of proteins and lipid containing peroxidation products of polyunsaturated fatty acids, which are formed in relation to oxidative stress, and aging process. Lipofuscin granules are highly seen in postmitotic cells and also characterized as undigested inclusion of amyloid proteins and other proteins due to lysosomal dysfunction in aged and diseased muscle and in the central nervous system [21–22].

Several papers reported that oxidative stress is implicated as a pertinent factor involved in pathogenesis of dystrophin-mutated muscular dystrophies [23,24]. In a severe DMD, dystrophin deficiency is proposed to cause profound oxidative damage, which may induce muscle necrosis that is thought to trigger the necrosis-regeneration necessary for renewal of myofibers. In contrast, in a milder BMD, although oxidative stress presumably is present at a lower level, it may lead to chronic accumulation of oxidized proteins and lipids in the absence of active necrosis and regeneration. We can only speculate that the myofibers in the BMD patients may mimic senescent status in which cellular homeostasis are slowed down. The presence of chronic myopathic changes, composed of myofiber inclusions, fiber atrophy and RV formation in BMD may reflect a long-standing process as exposed to oxidative state.

Common Mechanism of RV Formation in BMD to those in DMRV and sIBM

We found that only polyUb proteins and p62 can be seen deposited even in some fibers without and with RV. These results imply that at first, polyUb proteins were accumulated and then they recruited p62 to induce selective autophagy, as observed in neurodegenerative diseases [25]. Although in this study, we did not identify the polyUb proteins, Henderson et al. reported that the internally deleted-dystrophin minigene constructs revealed no cooperative transition during thermal denaturation and significant protein aggregation, suggesting increased susceptibility to misfolding, instability and aggregation of internally deleted-dystrophin proteins [26]. Further experiments on the identified dystrophin mutants will be required to clarify this issue.

Despite induction of autophagic process by recruitment of p62, the polyUb proteins are degraded, then leading to accumulation of numerous numbers of autophagic vacuoles. Such findings of