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
原著論文
Matsuda C, Kameyama K, Suzuki A, Mishima W, Yamaji S, Okamoto H, Nishino I, Hayashi YK: Affixin activates Rac1 via βPIX in C2C12 myoblast. *FEBS Lett.* 582: 1189-1196, 2008
Shalaby S, Hayashi YK, Goto K, Ogawa M, Nonaka I, Noguchi S, Nishino I: Rigid spine syndrome caused by a novel mutation in four-and-a-half LIM domain 1 gene (FHL1). *Neuromuscul Disord.* 18: 959-961, 2008
Arimura T, Hayashi YK, Murakami T, Oya Y, Funabe S, Arikawa-Hirasawa E, Hattori N, Nishino I, Kimura A: Mutational Analysis of Fukutin Gene in Dilated Cardiomyopathy and Hypertrophic Cardiomyopathy. *Circ J.* 73: 158-161, 2009
Park YE, Hayashi YK, Goto K, Komaki H, Hayashi Y, Inuzuka T, Noguchi S, Nonaka I, Nishino I: Nuclear changes in skeletal muscle extend to satellite cells in autosomal dominant Emery-Dreifuss muscular dystrophy/limb-girdle muscular dystrophy 1B. *Neuromuscul Disord.* 19: 29-36, 2009
Shalaby S, Hayashi YK, Nonaka I, Noguchi S, Nishino I: Novel FHL1 mutations in fatal and benign reducing body myopathy. *Neurology.* 72: 375-376, 2009
Murakami T, Hayashi YK, Ogawa M, Noguchi S, Campbell KP, Togawa M, Inoue T, Oka A, Ohno K, Nonaka I, Nishino I: A novel POMT2 mutation causes mild congenital muscular dystrophy with normal brain MRI. *Brain Dev.* 18: [in press]

総　説
Astejada MN, Goto K, Nagano A, Ura S, Noguchi S, Nonaka I, Nishino I, Hayashi YK: Emerinopathy and laminopathy clinical, pathological and molecular features of muscular dystrophy with nuclear envelopathy in Japan. *Acta Myol.* 26: 159-164, 2007

Ⅳ.　研究成果の刊行物・別刷

FEBS Letters 582 (2008) 1189–1196

Affixin activates Rac1 via βPIX in C2C12 myoblast

Chie Matsuda[a,b,*], Kimihiko Kameyama[a], Atsushi Suzuki[c], Wataru Mishima[d], Satoshi Yamaji[d], Harumasa Okamoto[a], Ichizo Nishino[b], Yukiko K. Hayashi[b]

[a] *Neuroscience Research Institute, AIST, Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan*
[b] *Department of Neuromuscular Research, National Institute of Neuroscience, NCNP, Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan*
[c] *Department of Molecular Biology, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan*
[d] *Department of Internal Medicine and Clinical Immunology, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan*

Received 25 December 2007; accepted 31 January 2008

Available online 4 March 2008

Edited by Berend Wieringa

Abstract Affixin/β-parvin is an integrin-linked kinase (ILK)-binding focal adhesion protein highly expressed in skeletal muscle and heart. To elucidate the possible role of affixin in skeletal muscle, we established stable C2C12 cell line expressing T7-tagged human affixin (C2C12-affixin cells). Exogenous expression of affixin promotes lamellipodium formation where affixin, ILK αp21-activated kinase (PAK)-interactive exchange factor (PIX) and βPIX accumulate. The association of affixin and βPIX was confirmed by immunoprecipitation and pull down assay. In C2C12-affixin cells, an increased level of activated Rac1 but not Cdc42 was observed, and mutant βPIX lacking guanine nucleotide exchange factor activity inhibited lamellipodium formation. These results suggest that affixin is involved in reorganization of subsarcolemmal cytoskeletal actin by activation of Rac1 through α and βPIXs in skeletal muscle.

Structured summary:
MINT-6179203, MINT-6179212, MINT-6178859, MINT-6178812, MINT-6178832, MINT-6178843:
Affixin (uniprotkb:Q9HBI1) *physically interacts* (MI:0218) with *βpix* (uniprotkb:Q9ES28) by *coimmunoprecipitation* (MI:0019)
MINT-6179221:
Affixin (uniprotkb:Q9HBI1) *physically interacts* (MI:0218) with *αpix* (uniprotkb:Q8K4I3) by *coimmunoprecipitation* (MI:0019)
MINT-6178962, MINT-6178983:
Affixin (uniprotkb:Q9HBI1) *physically interacts* (MI:0218) with *βpix* (uniprotkb:Q9ES28) by *pull-down* (MI:0096)
MINT-6179002, MINT-6179021:
Affixin (uniprotkb:Q9HBI1) *binds* (MI:0407) *βpix* (uniprotkb:Q9ES28) by *pull-down* (MI:0096)
MINT-6179039:
PAK1 (uniprotkb:Q13153) *physically interacts* (MI:0218) with *Rac1* (uniprotkb:P63001) by *pull-down* (MI:0096)
MINT-6179054:
PAK1 (uniprotkb:Q13153) *physically interacts* (MI:0218) with *Cdc42* (uniprotkb:P70766) by *pull-down* (MI:0096)
MINT-6178790:
Affixin (uniprotkb:Q9HBI1), *αpix* (uniprotkb:Q8K4I3) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-6178760:
Affixin (uniprotkb:Q9HBI1) and *βpix* (uniprotkb:Q9ES28) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-6178801:
Affixin (uniprotkb:Q9HBI1) and *dysferlin* (uniprotkb:Q9ESD7) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-6178779:
Affixin (uniprotkb:Q9HBI1) and *ILK* (uniprotkb:O55222) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

Keywords: Affixin/β-parvin; Lamellipodia; βPIX; Cytoskeletal actin

1. Introduction

Affixin/β-parvin (affixin) [1,2] is one of family of parvin family together with α-parvin/actopaxin/CH-ILKBP [1,3,4], and γ-parvin [1]. Parvins contain two caponin-homology (CH) domains and are known to have important role in focal adhesion, cell spreading and motility [5]. Our previous results revealed that affixin associates with αp21-activated kinase (PAK)-interactive exchange factor (PIX)/ARHGEF6/Cool-2 (αPIX) at the tips of lamellipodia of motile cells and transmits integrin-ILK signals which activate Cdc42 and Rac1, small Rho GTPases [6]. We also showed affixin directly binds to α-actinin which has a crucial role in reorganization of cytoskeletal actin [7]. Affixin is a protein highly expressed in skeletal muscle, and mainly localizes at sarcolemma [2]. We previously reported reduced sarcolemmal staining of affixin in dysferlin deficient skeletal muscles, and confirmed the association between affixin and dysferlin by an immunoprecipitation study [8]. Dysferlin is a sarcolemmal protein and its deficiency causes Miyoshi myopathy and limb girdle muscular dystrophy type 2B [9,10]. Based on the observation of

*Corresponding author. Fax: +81 29 861 6482.
E-mail address: c-matsuda@aist.go.jp (C. Matsuda).

Abbreviations: ILK, integrin-linked kinase; PIX, PAK-interactive exchange factor; C2C12-affixin, stable C2C12 cell line expressing T7-tagged human affixin; GEF, guanine nucleotide exchange factor; GST, gulutathione S-transferase; CH, caponin-homology; DH, Dbl-homology; PH, pleckstrin-homology; PAK, p21-activated kinase; CHO, Chinese hamster ovary

0014-5793/$34.00
doi:10.1016/j.febslet.2008.01.064

dysferlin accumulation at wounded sarcolemmal sites, dysferlin is suggested to have an important role in Ca^{2+}-induced membrane repair [11]. These results imply participation of affixin in membrane repair process of skeletal muscle together with dysferlin, although the precise biological function of affixin in skeletal muscle is not yet clear.

In this study, we established stable C2C12 myoblast cell lines expressing human affixin (C2C12-affixin cells) to elucidate the possible role of affixin in skeletal muscle. The C2C12 myoblast is derived from mouse satellite cell and widely used as an in vitro model for skeletal muscle [12]. Here we show that exogenous overexpression of affixin promotes lamellipodium formation. In the C2C12-affixin cells, affixin is co-localized with βPIX/ARHGEF7/Cool-1 (βPIX) at lamellipodia together with αPIX, ILK and dysferlin. βPIX is a close homolog of αPIX and known to induce membrane ruffling [13]. The interaction of affixin with βPIX is confirmed by immunoprecipitation and pulldown assay. The level of activated Rac1 increased in the C2C12-affixin cells compared to C2C12 cells. Lamellipodium formation of the C2C12-affixin cells is suppressed by transfection of mutant αPIX or βPIX lacking guanine nucleotide exchange factor (GEF) activity. These results suggest an important role of affixin in subsarcolemmal actin reorganization by activation of Rac1 through α and βPIXs in skeletal muscle.

2. Materials and methods

2.1. Cell culture and establishment of stable transfectant

C2C12 myoblasts and COS-7 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. C2C12 cells were transfected with T7-tagged human affixin cDNA subcloned into pcDNA3.1 (Invitrogen) using Lipofectamine 2000 (Invitrogen). All construct sequences were verified with DNA sequencing using ABI PRISM 310 (Applied Biosystems). The cells expressing T7-tagged human affixin were selected in growth media with 1 mg/ml G418 (Invitrogen). The surviving colonies were isolated and separately amplified. Cell line maintenance was performed with 0.5 mg/ml G418.

2.2. Antibodies

Monoclonal antibody against human dysferlin (NCL-Hamlet-2) was purchased from Novocastra. Rabbit polyclonal antibody against human affixin was previously characterized [2]. Rabbit polyclonal antibody against human αPIX was generated as described previously by Manser et al. [13]. We confirmed that affinity-purified anti-αPIX antibody did not cross-react with human βPIX expressed in COS-7 cells. Anti-ILK monoclonal antibody (Upstate Biotechnology), anti-βPIX polyclonal antibody (Chemicon International), anti-βPIX monoclonal antibody (BD Transduction Laboratories), anti-T7 polyclonal antibody (Omni-probe; Santa Cruz), anti-T7 monoclonal antibody (Novagen), anti-HA rat monoclonal antibody (3F10; Roche), anti-HA monoclonal antibody (262K; New England Biolabs), anti-$(His)_6$ polyclonal antibody (His-probe; Santa Cruz), anti-Rac1 (23A8; Upstate) and Cdc42 (clone 44; BD Transduction Laboratories) were used.

2.3. Immunofluorescent analysis

C2C12-affixin cells seeded on coverslips were fixed for 15 min in 2% paraformaldehyde in PBS and then permeabilized for 10 min in 0.1% Triton X-100 in PBS. For double immunolabeling with anti-affixin and anti-dysferlin antibodies, cells were fixed at −20 °C for 10 min in 100% methanol. Coverslips were blocked with 5% goat serum–2% BSA in PBS and then incubated with primary antibodies for double labelling. Immunolabeling was detected with goat anti-rabbit IgG conjugated to FITC and goat anti-mouse IgG conjugated to Cy3 antibodies (Jackson Immunoresearch Laboratories). Cells were observed with a confocal laser-scanning microscope (LSM5 PASCAL, Carl Zeiss).

2.4. Immunoprecipitation assay

C2C12 and C2C12-affixin cells were lysed in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and Complete (Roche). The lysates precleared with Protein A/G-agarose (ImmunoPure, PIERCE) were incubated with anti-affixin, αPIX and βPIX polyclonal antibodies and then Protein A/G-agarose was added before an additional incubation. Immunoprecipitated proteins were dissociated from beads by boiling in sample buffer and resolved by SDS–PAGE. Immunoblotting was performed as previously described [14].

COS-7 cells were co-transfected with T7-tagged wild type or deletion mutant human affixin (RP1 and RP2) and HA-tagged human βPIX using FuGENE 6 (Roche), and lysed for immunoprecipitation after 48 h. Affixin deletion mutants were generated as previously described [2,6]. Human βPIX cDNA was cloned from KIAA0142 gifted by Dr. T. Nagase (Kazusa DNA Research Institute, Japan). Immunoprecipitation was performed as outlined above except that anti-T7 (Novagen) and anti-HA (New England BioLabs) antibodies were used.

2.5. Gulutathione S-transferase (GST)-βPIX pulldown assay

The GST-βPIX proteins used for the pulldown assay were as follows: GST-SH3 (corresponding to amino acids of human βPIX 6-65), GST-Dbl-homology (DH) (aa 93–273), GST-pleckstrin-homology (PH) (aa 295–400), GST-CC (aa 586–638). cDNA fragments of these domains were amplified by PCR and subcloned into pGEX-5X-3 (GE Healthcare). GST fusion proteins expressed in BL21 were purified and bound to glutathione Sepharose 4B (GE Healthcare). The COS-7 cells overexpressing T7-tagged human affixin were lysed in the same lysis buffer used for immunoprecipitation. Precleared lysates were diluted with Buffer A (10 mM Tris–HCl, pH 8.0 and 0.1% Tween20) [15] and incubated with fusion protein bound to glutathione Sepharose 4B. After five washes in PBS, sample buffer was added to the beads and boiled for 5 min. Bound proteins were resolved by SDS–PAGE and subjected to immunoblotting using anti-T7 polyclonal antibody.

2.6. $(His)_6$-tagged affixin pulldown assay

cDNAs of deletion mutant affixin (RP1 and RP2) were amplified by PCR and subcloned into pET32a (Novagen). The $(His)_6$-tagged RP1 and RP2 were expressed in BL21DE3pLys(S), purified using Ni-NTA Spin kit (QIAGEN) and dialyzed against PBS. The pulldown assay using GST-βPIX fusion proteins and $(His)_6$-tagged RP1 or RP2 was performed as above.

2.7. Small GTPase activation assay

C2C12 and C2C12-affixin cells were grown until 60–70% confluence. Cells were lysed in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA and 2% glycerol. Lysates were incubated with glutathione Sepharose 4B conjugated with GST-p21-binding (CRIB) domain (residues 67–150) of human PAK-1 [16]. After three washes in the lysis buffer, the beads were resuspended in sample buffer and boiled for five minutes. Bound Rac1 and Cdc42 were separated on SDS–PAGE and subjected to immunoblotting.

2.8. Introduction of dominant-negative PIX into C2C12-affixin cells

Double mutation of βPIX (L238R, L239S) was introduced by PCR using appropriate internal primers. These two leucine residues were highly conserved among Dbl family member and shown to be essential for GEF activity [17]. βPIX and mutant βPIX (L238R, L239S) were subcloned into pSRD4-HA for transient expression. The constructs for transient expression of wild type αPIX and dominant negative αPIX (L383R, L384S) were as previously described [6]. C2C12-affixin cells were transfected with wild type or mutant PIX using Lipofectamine 2000. After 48 h, cells were fixed and immunolabeled as above.

3. Results

3.1. Immunofluorescent analysis of the C2C12-affixin cells

To investigate the possible role of affixin in skeletal muscle, stable C2C12 cell lines constitutively expressing T7-tagged human affixin were established. Expression of T7-tagged human affixin in C2C12-affixin cells was confirmed by immunoblot using anti-T7 antibody (Fig. 2). The expression level of total

affixin of C2C12-affixin cells was slightly higher than original C2C12 cell on immunoblotting using anti-affixin antibody (data not shown). Exogenous expression of T7-affixin in the C2C12 cells induced lamellipodia (Fig. 1). Only 10–20% of original C2C12 myoblasts showed lamellipodium formation, while more than half of the C2C12-affixin cells formed lamellipodia without any stimulation (Fig. 1). Formation of lamellipodia was confirmed by labelling F-actin with rhodamine–phalloidin.

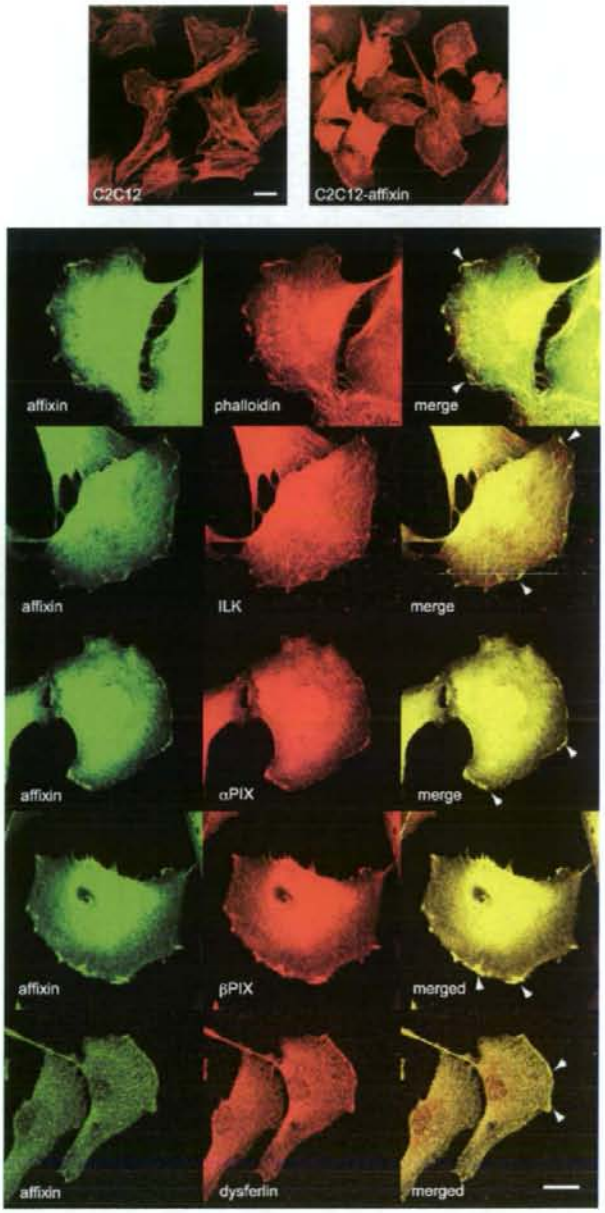

Fig. 1. Immunofluorescence analysis of stable C2C12 cells expressing human affixin. Lamellipodium formation was confirmed by rhodamine–phalloidin labelling. Antibodies were applied in five double-staining combinations: anti-affixin and phalloidin; anti-affixin and ILK; anti-T7 and αPIX; anti-affixin and βPIX and anti-affixin and dysferlin. Exogenous affixin was labelled with anti-T7 monoclonal antibody. Affixin co-localizes with ILK, αPIX, βPIX and dysferlin lamellipodium tips (arrowheads) in the C2C12-affixin cells. Scale bar, 20 μm.

As shown in Fig. 1, affixin accumulated and co-localized with F-actin at the tips of lamellipodia (Fig. 1). Transient expression of affixin without T7-tag promoted the lamellipodium formation of C2C12 cells as T7-tagged affixin (data not shown). Exogenous affixin was expressed in both the cytoplasm and at the tips of lamellipodia as C2C12-endogenous affixin (data not shown). We have previously shown the co-localization of affixin and ILK, a binding partner of affixin, at focal adhesion and at the tips of leading edge in Chinese hamster ovary (CHO) cells [2], and at sarcolemma of human skeletal muscle fibers [8]. In C2C12-affixin cells, ILK was enriched and co-localized with affixin in lamellipodia (Fig. 1) as original C2C12 cells. Cytoplasmic affixin in the C2C12-affixin cells was partially co-localized with ILK.

αPIX, a binding partner of affixin co-localizes with exogenous affixin at the tips of lamellipodia in 3Y1 cells (rat fibroblasts) [6] and CHO-K1 cells [18]. In the C2C12-affixin cells, endogenous αPIX accumulated intensely at the tips lamellipodia and co-localized with affixin (Fig. 1), while diffuse fine cytoplasmic granular staining of αPIX without lammellipodium accumulation was observed in the original C2C12 cells (data not shown). The C2C12-affixin cells also showed intense staining of βPIX, a homolog and a binding partner of αPIX [18,19], at lamellipodia, suggesting a possible association of affixin and βPIX. The co-localization of βPIX and affixin was observed at lamellipodia of original C2C12 cells (data not shown). Cytoplasmic α and βPIXs were also co-localized with affixin in the C2C12 cells. We then examined subcellular localization of dysferlin, a binding partner of affixin, in C2C12-affixin cells. As we reported earlier, cytoplasmic granular staining of dysferlin with no sarcolemmal accumulation was observed in the undifferentiated C2C12 cells [8]. In the C2C12-affixin cells, dysferlin accumulated and co-localized with affixin at lamellipodia. These results show that affixin co-localizes with ILK, αPIX, βPIX and dysferlin at the tips lamellipodia in the C2C12-affixin cells.

3.2. Analysis of the association between affixin and βPIX by coimmunoprecipitation assay

To define possible association between affixin and βPIX, immunoprecipitation was performed using the C2C12-affixin and original C2C12 cells. As shown in Fig. 2A, affixin was specifically co-immunoprecipitated with anti-βPIX antibody and reciprocally, βPIX was specifically co-immunoprecipitated by anti-affixin antibody in both C2C12-affixin and original C2C12 cells. There is no significant quantitative difference in co-immunoprecipitated affixin, α and βPIXs between C2C12-affixin and original C2C12 cells. Further, αPIX was specifically co-immunoprecipitated by anti-affixin [6] and anti-βPIX [18,19] antibodies in C2C12-affixin and original C2C12 cells as previously reported. Affixin or βPIX was not co-immunoprecipitated by control rabbit IgG.

To identify the region within affixin that interacts with βPIX, immunoprecipitation was also performed using COS-7 cells transiently co-transfected with full-length or deletion mutants [2] of T7-human affixin (Fig. 2A) and HA-human βPIX. As shown in Fig. 2B, the full-length T7-affixin was co-immunoprecipitated by anti-HA antibody. Reciprocally, HA-βPIX was specifically co-immunoprecipitated by anti-T7 antibody, while control mouse IgG was not. Affixin-RP1 containing N-terminal CH domain (CH1) was co-immunoprecipitated by

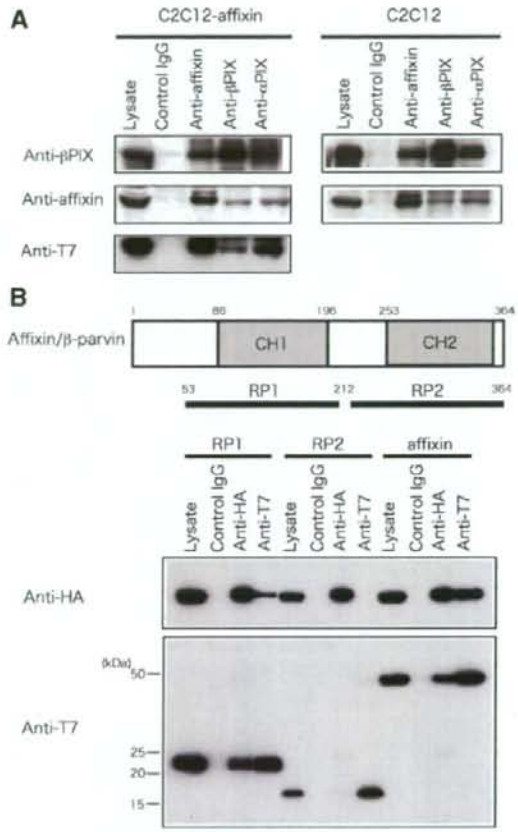

Fig. 2. (A) Confirmation of the association of affixin and βPIX. Cell lysates form the C2C12-affixin and C2C12 cells were immunoprecipitated with anti-affixin, anti-βPIX and anti-αPIX antibodies. Immunoprecipitates were subjected to immunoblotting with the same antibodies used for immunoprecipitation. Affixin was specifically co-immunoprecipitated by anti-βPIX antibody and *vice versa*. Co-immunoprecipitation of T7-tagged exogenous affixin was confirmed by probing with anti-T7 antibody. As reported previously, αPIX was specifically co-immunoprecipitated by anti-affixin and anti-βPIX antibodies. (B) Identification of βPIX-binding domain of affixin by immunoprecipitation assay. T7-tagged wild type or deletion mutants of affixin and HA-tagged βPIX were co-expressed in COS-7 cells. A schema of deletion mutants of affxin is shown at the top. Immunoprecipitation was performed with anti-T7 and anti-HA antibodies. βPIX was co-immunoprecipitated with wild type affixin and RP1 and *vice versa*, but not with RP2.

HA-βPIX (Fig. 2B), while affixin-RP2 containing C-terminal CH domain (CH2) was not. These and previous reported findings [6] suggest that affixin can interact with both α and βPIXs via CH1 domain.

3.3. Identification of the binding domain of βPIX to affixin by pulldown assay

To identify affixin-binding domain of βPIX, a pulldown assay was performed. The lysates from COS-7 cells overexpress-

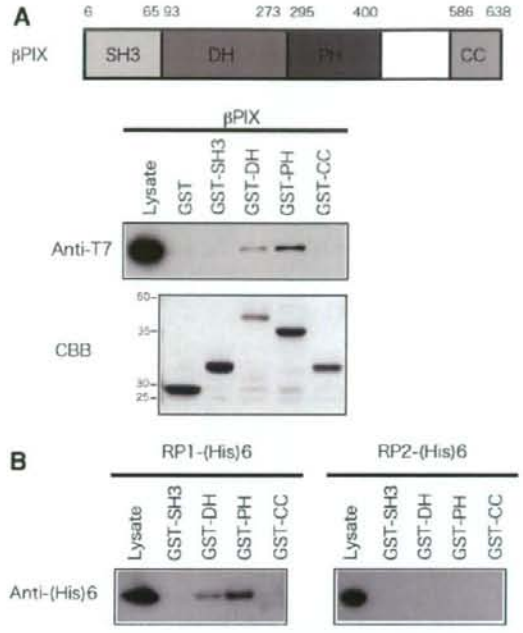

Fig. 3. Identification of affixin-binding domain in βPIX by pulldown assay. (A) Cell lysates from COS-7 cells overexpressing T7-tagged affixin were incubated with glutathione Sepharose 4B beads bound to GST, GST-SH3, GST-DH, GST-PH and GST-CC. Domain structures of βPIX are shown at the top. After overnight incubation at 4 °C, the beads were washed and bound proteins were subjected to immunoblotting with anti-T7 polyclonal antibody. A band at 50 kDa, which corresponds to T7-affixin, was detected in GST-DH and GST-PH. GST fusion proteins used for the pulldown assay were stained with coomasie brilliant blue. GST, GST-SH3, GST-DH, GST-PH and GST-CC domains have molecular masses of 26, 32.8, 46.6, 38 and 32.3 kDa, respectively. (B) Bacterially expressed $(His)_6$-tagged RP1 and RP2 were purified and incubated with GST-βPIX domains as described above. The proteins bound to GST fusion proteins were subjected to immunoblotting using anti-$(His)_6$ antibody.

ing T7-affixin were incubated with GST fusion proteins carrying each domain of human βPIX bound to glutathione Sepharose 4B. Fig. 3A shows that the DH and PH domains of βPIX can interact with T7-affixin, whereas the SH3 and CC domains of βPIX failed. PH domain of βPIX showed higher affinity to T7-affixin than DH domain of βPIX. To examine whether affixin-RP1 directly binds to DH and PH domains of βPIX, pulldown assay was performed using bacterially expressed $(His)_6$-tagged RP1 instead of cell lysate. As shown in Fig. 3B, $(His)_6$-tagged RP1 was bound to DH and PH domains of βPIX, whereas $(His)_6$-tagged RP2 failed to interact with any domain of βPIX. PH domain of βPIX showed higher affinity to $(His)_6$-tagged RP1 than DH domain of βPIX. These results were consistent with the result of a pulldown assay using cell lysates and suggest direct interaction between affixin and βPIX.

3.4. Rac1 but not Cdc42 is activated in C2C12-affixin cells

The reorganization of cytoskeletal actin is regulated by Rho family small GTPases. βPIX is known as a specific GEF for

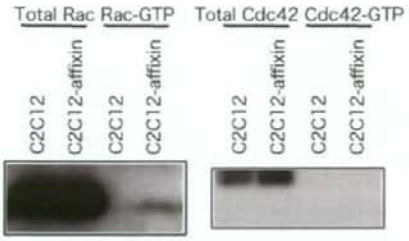

Fig. 4. Pulldown assay of active Rac1 and Cdc42. C2C12-affixin and C2C12 cells were lysed and incubated with glutathione Sepharose 4B conjugated with GST-p21-binding (CRIB) domain (residues 67–150) of human PAK-1. The CRIB domain specifically associates with Rac-GTP and Cdc42-GTP. The total amounts of Rac1 and Cdc42 were assessed by the immunoblotting using the cell lysates.

Rac1/Cdc42 and induces membrane ruffling [13]. To examine whether Rac1/Cdc42 is activated in the C2C12-affixin cells, a pulldown assay was performed using GST-fusion protein derived from PAK, the effector protein of Rac1 and Cdc42. Cell lysates from C2C12-affixin and C2C12 cells were incubated with GST-p21 binding domain of PAK bound to glutathione Sepharose 4B. This analysis revealed increased level of Rac1-GTP in the C2C12-affixin cells (Fig. 4). There was no significant difference in Cdc42-GTP. The total expression levels of Rac1 and Cdc42 protein in the C2C12-affixin were equivalent to the C2C12 cells. These results indicate that the exogenous expression of affixin can activate Rac1 but not Cdc42 in C2C12 cells.

3.5. Dominant-negative βPIX suppresses lamellipodium formation in the C2C12-affixin cells

Activation of Rac1 is mediated by GEFs including βPIX [20]. To examine the involvement of βPIX in lamellipodium formation observed in the C2C12-affixin cells, the HA-tagged dominant negative mutant of βPIX were introduced into the C2C12-affixin cells. Exogenous βPIX and affixin were labelled with anti-HA and anti-T7 antibodies, respectively. As shown in Fig. 5, the C2C12-affixin cells overexpressing wild type βPIX form lamellipodia as observed in the C2C12-affixin cells without transfection. In contrast, dominant negative mutant of βPIX (L238R, L239S) suppressed the lamellipodium formation in the C2C12-affixin cells. The association of dominant negative βPIX and affixin was confirmed by an immunoprecipitation study using COS-7 cells (data not shown). Similar inhibition of lamellipodium formation was observed in the C2C12-affixin cells transfected with dominant negative mutant of αPIX.. In C2C12-affixin cells transiently transfected with dominant negative mutants of PIX, expression level of affixin, α and βPIXs was equivalent to C2C12-affixin cells without transfection on immunoblot (data not shown). These results suggest that both α and βPIXs are necessary for the lamellipodium formation in the C2C12-affixin cells.

4. Discussion

Lamellipodium is the dynamic actin-based structure and its formation is mediated by the activation of Rho family small GTPases and their effector proteins. Small Rho GTPases are activated by GEFs, which catalyze exchange of GDP for

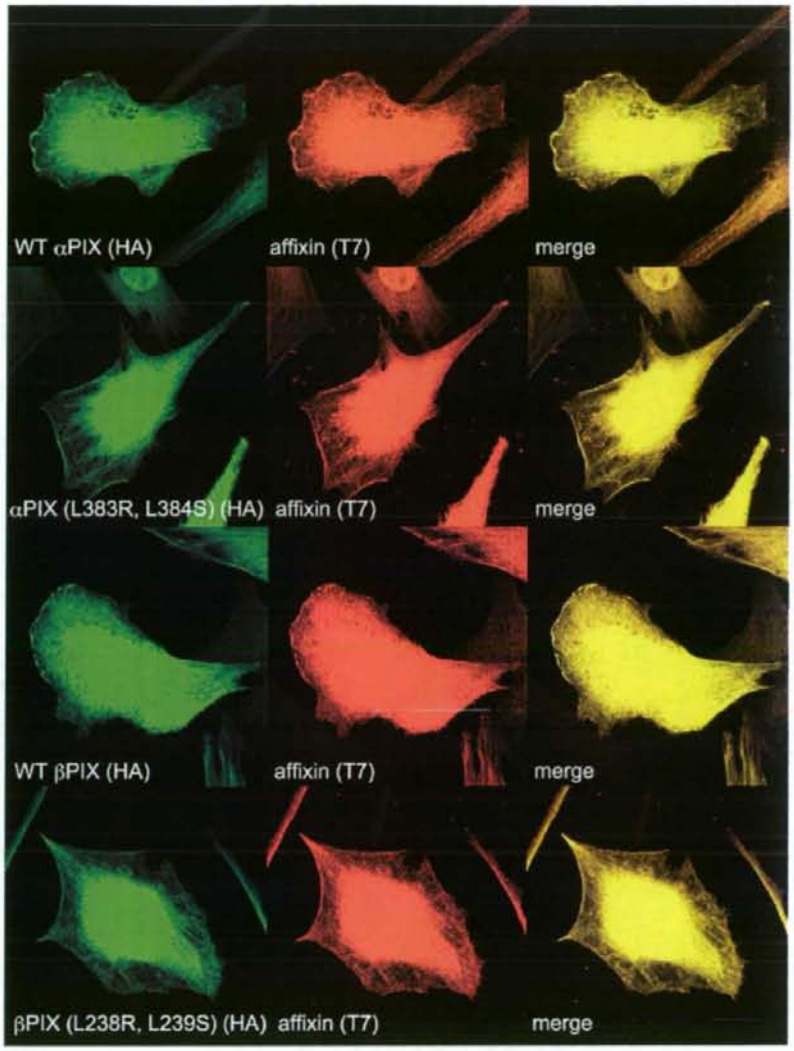

Fig. 5. Immunofluorescence analysis of the C2C12-affixin cells transformed with the dominant negative mutants of PIXs. The C2C12-affixin cells were transfected with wild type or dominant negative mutants of PIXs, αPIX (L383R, L384S) and βPIX (L238R, L239S). Exogenous PIX and affixin were simultaneously immunolabeled with anti-HA and anti-T7 antibodies, respectively. Scale bar, 20 μm.

GTP. GEFs are regulated by protein–protein interaction, oligomerization and relief of intramolecular inhibitory sequence [21]. More than 60 GEFs including PIXs have been identified in human genomic sequences [22]. βPIX expression levels are highest in skeletal muscle by Northern blot analysis using KIAA clones as probes (KIAA0142, http://www.kazusa.or.jp/huge/gfimage/northern/html/KIAA0142.html).

In C2C12 cells, exogenous expression of affixin induced prominent lamellipodia. Accumulated βPIX, together with ILK was observed at the tips of lammelipodia and co-localized with affixin. The interaction of affixin and βPIX was confirmed by immmunoprecipitation and pulldown assays. These data suggest that affixin could promote reorganization of subsarcolemmal actin cytoskeleton associated with accumulation of GEFs.

We previously demonstrated that Madin-Darby canine kidney cells overexpressing CH1 domain of affixin formed membrane protrusions, while control cells transfected with lacZ showed a cobble-like morphology [6]. This CH1-induced reorganization of cytoskeletal actin is mediated by the activation of both Rac1 and Cdc42 through αPIX. Interestingly, only Rac1 but not Cdc42 is activated in the C2C12-affixin cells used for this study. αPIX has been reported to contain Rac-specific interaction domain at C-terminus of PH domain [23]. When

αPIXs form a homodimer, the DH domain of one molecule and Rac-specific interaction domain of another molecule work together and bind Rac specifically, whereas monomeric αPIX can interact with Cdc42 as well as Rac. Despite the lack of a Rac-specific interaction domain, βPIX binds directly and activates Rac1 specifically but not Cdc42 in human embryonal kidney 293 cells [24]. Previous study using yeast two hybrid system [18,19] and our immunoprecipitation results have shown that α and βPIX form heterodimers in the C2C12-affixin cells (Fig. 2A), although their specificity for small GTPase remains unclear. We have also shown that lamellipodium formation in the C2C12-affixin cells was inhibited by overexpression of dominant negative forms of α or βPIX (Fig. 5). From these results, we suspect that the DH domain of βPIX and the Rac-specific interacting domain of αPIX work together and activate specifically Rac1. Further analyses are needed to elucidate the precise regulation of GEF activity of both PIXs in C2C12-affixin cells.

Lamellipodium formation is essential for cell motility. In epithelial cell monolayer, the cells around the wounded edge form lamellipodia and activation of Rac1 but not Cdc42 or Rho is required for wound closure [25]. Similarly, activated satellite cells form lamellipodia and migrate around the injured lesion during skeletal muscle regeneration [26]. Very recently, dysferlin reportedly localized in the T-tubule system of differentiating C2C12 cells and was recruited to the wounded site [27]. Dysferlin is known to have an important role in skeletal muscle wound healing [11]. In response to the sarcolemmal injury of muscle fibers, dysferlin patch was formed around wounded sites for resealing in a calcium-dependent manner. Calcium-dependent membrane resealing is also reported in wounded *Xenopus* oocyte, where Cdc42 and RhoA are activated [28]. Membrane repair is composed of two process: plasma membrane resealing and reorganization of subsarcolemmal cytoskeleton [29]. Accumulation of dysferlin and activation of Rac1 via PIXs in the lamellipodia of C2C12-affixin cells may suggest the involvement of these molecules in sarcolemmal repair followed by cytoskeletal actin reorganization.

In conclusion, exogenous expression of affixin promotes lamellipodium formation in C2C12 myoblasts via activation of Rac1 by α and βPIXs. Reorganization of cytoskeletal actin mediated by affixin may be involved in the skeletal muscle dysferlin-related membrane repair system.

Acknowledgements: This work was supported by Grants-in-aid for Scientific Research (C.M.; C, 15590924, 18590966) (Y.K.H.; B, 18390265), and Grant-in-Aid for Exploratory Research (Y.K.H.; 50238135) from the Japan Society for the Promotion of Science, the Nakatomi Foundation (C.M.), the Research on Psychiatric and Neurological Diseases and Mental Health from Health and Labor Sciences Research Grants (Y.K.H. and I.N.), and the Research Grant for Nervous and Mental Disorders, from the Ministry of Health, Labour and Welfare (I.N.).

References

[1] Olski, T.M., Noegel, A.A. and Korenbaum, E. (2001) Parvin, a 42 kDa focal adhesion protein, related to the alpha-actinin superfamily. J. Cell Sci. 114, 525–538.
[2] Yamaji, S. et al. (2001) A novel integrin-linked kinase-binding protein, affixin, is involved in the early stage of cell–substrate interaction. J. Cell Biol. 153, 1251–1264.
[3] Nikolopoulos, S.N. and Turner, C.E. (2000) Actopaxin, a new focal adhesion protein that binds paxillin LD motifs and actin and regulates cell adhesion. J. Cell Biol. 151, 1435–1448.
[4] Tu, Y., Huang, Y., Zhang, Y., Hua, Y. and Wu, C. (2001) A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. J. Cell Biol. 153, 585–598.
[5] Sepulveda, J.L. and Wu, C. (2006) The parvins. Cell. Mol. Life Sci. 63, 25–35.
[6] Mishima, W. et al. (2004) The first CH domain of affixin activates Cdc42 and Rac1 through alphaPIX, a Cdc42/Rac1-specific guanine nucleotide exchanging factor. Gene Cell 9, 193–204.
[7] Yamaji, S. et al. (2004) Affixin interacts with alpha-actinin and mediates integrin signaling for reorganization of F-actin induced by initial cell–substrate interaction. J. Cell Biol. 165, 539–551.
[8] Matsuda, C. et al. (2005) Dysferlin interacts with affixin (beta-parvin) at the sarcolemma. J. Neuropathol. Exp. Neurol. 64, 334–340.
[9] Liu, J. et al. (1998) Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. Nat. Genet. 20, 31–36.
[10] Bashir, R. et al. (1998) A gene related to *Caenorhabditis elegans* spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B. Nat. Genet. 20, 37–42.
[11] Bansal, D., Miyake, K., Vogel, S.S., Groh, S., Chen, C.C., Williamson, R., McNeil, P.L. and Campbell, K.P. (2003) Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature 423, 168–172.
[12] Yaffe, D. and Saxel, O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270, 725–727.
[13] Manser, E. et al. (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol. Cell 1, 183–192.
[14] Matsuda, C., Aoki, M., Hayashi, Y.K., Ho, M.F., Arahata, K. and Brown Jr., R.H. (1999) Dysferlin is a surface membrane-associated protein that is absent in Miyoshi myopathy. Neurology 53, 1119–1122.
[15] Sotgia, F. et al. (2000) Caveolin-3 directly interacts with the C-terminal tail of beta -dystroglycan. Identification of a central WW-like domain within caveolin family members. J. Biol. Chem. 275, 38048–38058.
[16] del Pozo, M.A., Price, L.S., Alderson, N.B., Ren, X.D. and Schwartz, M.A. (2000) Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. EMBO J. 19, 2008–2014.
[17] Hart, M.J., Eva, A., Zangrilli, D., Aaronson, S.A., Evans, T., Cerione, R.A. and Zheng, Y. (1994) Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. J. Biol. Chem. 269, 62–65.
[18] Rosenberger, G., Jantke, I., Gal, A. and Kutsche, K. (2003) Interaction of alphaPIX (ARHGEF6) with beta-parvin (PARVB) suggests an involvement of alphaPIX in integrin-mediated signaling. Hum. Mol. Genet. 12, 155–167.
[19] Koh, C.G., Manser, E., Zhao, Z.S., Ng, C.P. and Lim, L. (2001) Beta1PIX, the PAK-interacting exchange factor, requires localization via a coiled-coil region to promote microvillus-like structures and membrane ruffles. J. Cell Sci. 114, 4239–4251.
[20] Scita, G., Tenca, P., Frittoli, E., Tocchetti, A., Innocenti, M., Giardina, G. and Di Fiore, P.P. (2000) Signaling from Ras to Rac and beyond: not just a matter of GEFs. EMBO J. 19, 2393–2398.
[21] Schmidt, A. and Hall, A. (2002) Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Gene Dev. 16, 1587–1609.
[22] Venter, J.C. et al. (2001) The sequence of the human genome. Science 291, 1304–1351.
[23] Feng, Q., Baird, D. and Cerione, R.A. (2004) Novel regulatory mechanisms for the Dbl family guanine nucleotide exchange factor Cool-2/alpha-Pix. EMBO J. 23, 3492–3504.
[24] ten Klooster, J.P., Jaffer, Z.M., Chernoff, J. and Hordijk, P.L. (2006) Targeting and activation of Rac1 are mediated by the exchange factor beta-Pix. J. Cell Biol. 172, 759–769.

[25] Fenteany, G., Janmey, P.A. and Stossel, T.P. (2000) Signaling pathways and cell mechanics involved in wound closure by epithelial cell sheets. Curr. Biol. 10, 831–838.
[26] Bischoff, R. and Flanzini-Armstrong, C. (2004) Stellite and stem cells in muscle regeneration. Myology, 66–86.
[27] Klinge, L., Laval, S., Keers, S., Haldane, F., Straub, V., Barresi, R. and Bushby, K. (2007) From T-tubule to sarcolemma: damage-induced dysferlin translocation in early myogenesis. FASEB J.
[28] Benink, H.A. and Bement, W.M. (2005) Concentric zones of active RhoA and Cdc42 around single cell wounds. J. Cell Biol. 168, 429–439.
[29] Bement, W.M., Yu, H.Y., Burkel, B.M., Vaughan, E.M. and Clark, A.G. (2007) Rehabilitation and the single cell. Curr. Opin. Cell Biol. 19, 95–100.

Neuromuscular Disorders 18 (2008) 959–961

Contents lists available at ScienceDirect

Neuromuscular Disorders

journal homepage: www.elsevier.com/locate/nmd

Case report

Rigid spine syndrome caused by a novel mutation in four-and-a-half LIM domain 1 gene (*FHL1*)

Sherine Shalaby, Yukiko K. Hayashi*, Kanako Goto, Megumu Ogawa, Ikuya Nonaka, Satoru Noguchi, Ichizo Nishino

Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawahigashi-cho, Kodaira, Tokyo 187-8502, Japan

ARTICLE INFO

Article history:
Received 19 May 2008
Received in revised form 9 September 2008
Accepted 17 September 2008

Keywords:
Four-and-a-half LIM domain 1 (*FHL1*)
Rigid spine syndrome
Reducing body

ABSTRACT

Four-and-a-half LIM domain 1 gene (*FHL1*) has recently been identified as the causative gene for reducing body myopathy (RBM), X-linked scapuloperoneal myopathy (SPM) and X-linked myopathy with postural muscle atrophy (XMPMA). Rigid spine is a common clinical feature of the three diseases. We searched for *FHL1* mutations in eighteen patients clinically diagnosed as rigid spine syndrome (RSS). We identified one RSS patient with *FHL1* mutation. Reducing bodies were observed in few fibers of the patient's muscle sample. Amount of FHL1 protein was decreased on immunoblotting. In conclusion, *FHL1* can be one of the causative genes for RSS.

1. Introduction

FHL1, four-and-a-half LIM domain 1 is a 32 kDa protein which is highly expressed in skeletal muscle with intermediate expression in the heart [1]. LIM domains are a cysteine-rich double zinc finger protein-binding motif denoted by the sequence (CX2–CX17–19HX2C)X2(CX2CX16–20CX2(H/D/C)) and mediate interactions with transcription factors and cytoskeletal proteins. LIM domain proteins play critical roles in tissue differentiation and cytoskeletal integrity, respectively. FHL1 was implicated in many cellular functions; (1) α5β1-integrin-dependent myocyte elongation [2], (2) regulation of myosin filament formation and sarcomere assembly by binding to myosin-binding protein C [3], and (3) modulation of Notch signalling pathway through interaction of FHL1C (one of the splicing isoforms of FHL1) with transcription factor RBP-J and RING1 [4].

Recently, mutations in *FHL1* have been identified in patients with RBM [5], SPM [6] and XMPMA [7]. We have also identified mutations in *FHL1* in all RBM patients we reported previously, and confirmed that *FHL1* is the causative gene for RBM (unpublished data). Clinical picture of RBM patients varies from congenital lethal form to benign childhood and adult forms. However, four out of the six RBM families reported to date show rigid spine [5,8]. In addition rigid spine was reported in SPM families [9] and was also seen in the British and Italian–American families reported as XMPMA [7]. This finding suggests that rigid spine is a common clinical feature of patients with *FHL1* mutations.

Here we found a patient with rigid spine syndrome (RSS) harboring a mutation in *FHL1* among 18 patients clinically diagnosed as RSS.

2. Case report

The patient is a 16-year-old male who was a good runner during his childhood. He was first noted to have scoliosis on a routine medical examination when he was 13 years old. Gradually, his walking and running speed became slower, and hip muscle atrophy was noted. Two years late he started experiencing difficulty in bending his body and difficulty in neck flexion. He could not stand on one foot. By the age of 16 years, bilateral hip and thigh muscle atrophy was prominent. On examination, he showed muscle weakness and atrophy in the sternomastoid, trapezius, paravertebral, pelvic girdle and proximal lower limb muscles. Winging of scapula and Gowers' sign were observed. Funnel chest and joint contractures in neck, spine, hip and ankle joints were seen. He walked slouchingly and his left leg was slightly lagged and outward rotated. Serum creatine kinase level was mildly elevated and respiratory functions were mildly impaired. His elder brother showed mild scoliosis but not rigid spine or muscle weakness. His father had IRBBB while his mother was healthy.

Genomic DNA was isolated from peripheral lymphocytes using a standard technique after obtaining informed consent. Seven sets of primers were used to amplify genomic fragments of *FHL1*. All

* Corresponding author. Tel.: +81 42 346 1712; fax: +81 42 346 1742.
E-mail address: hayasi_y@ncnp.go.jp (Y.K. Hayashi).

0960-8966/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.nmd.2008.09.012

exons and their flanking intronic regions of *FHL1* were directly sequenced using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems). We identified a hemizygous in-frame nine base-pair (bp) deletion mutation at c.451–459delGTGACTTGC (p.151–153delVTC) of *FHL1* in this patient. A total 250 controls and the other 17 RSS patients did not carry the mutation in *FHL1*. Genetic analysis of other family members including the elder brother was not allowed.

Biopsied muscle specimen was frozen in isopentane cooled in liquid nitrogen. Serial 10 μm cryostat sections were stained with haematoxylin and eosin (HE), modified Gomori trichrome (mGt) and a battery of histochemical methods. Menadione–nitroblue tetrazolium (NBT) staining in the absence of the substrate α-glycerophosphate was also performed to detect reducing bodies (RBs). Histological analyses of muscle showed marked variation in fiber size and fibers with rimmed vacuoles. Only a limited number of fibers contained RBs. These abnormal fibers detected were localized in focal areas of the muscle specimen (Fig. 1A and B).

Immunohistochemical analysis revealed diffusely increased FHL1 staining in some muscle fibers. The strong FHL1 staining was observed in both types of fibers as seen in serial sections stained by slow type of myosin heavy chain (MHC-slow) (Fig. 1C and D). Protein amount of FHL1 by immunoblotting analysis was significantly reduced in the patient muscle when compared to normal control after normalization to actin amount (Fig. 2).

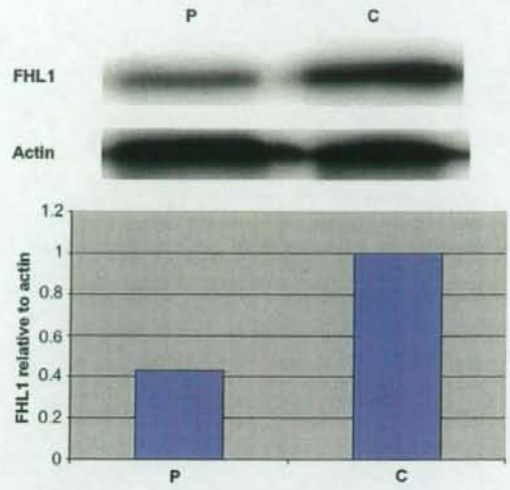

Fig. 2. Immunoblotting analysis of FHL1. Amount of FHL1 in biopsied muscle from the RSS patient show significant reduction compared to actin.

3. Discussion

The term *rigid spine syndrome* was first proposed by Dubowitz to highlight the essential clinical problem seen in myopathy with prominent spinal rigidity [10]. Nevertheless, spinal rigidity is not a specific finding as it is a characteristic feature in Emery–Dreifuss muscular dystrophy, Bethlem myopathy, and in selenoprotein related myopathies. In addition it has also been reported in other congenital myopathies and muscular dystrophies. Patients with *FHL1* mutations also show spinal rigidity [5,7,9].

Here we identified a RSS patient with a novel mutation in *FHL1*. The mutation affects a cysteine residue in the second LIM domain of FHL1 similar to all mutations causing RBM [5].

The most important feature to differentiate RSS from other muscular diseases associated with spinal rigidity is the limitation of flexion of the cervical and dorsolumbar spine in absence of

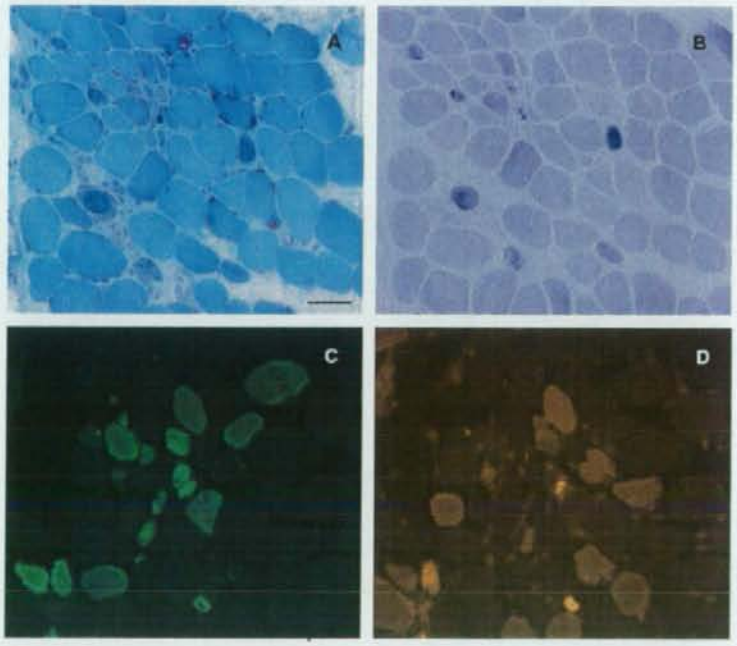

Fig. 1. Muscle pathology. (A) Intracytoplasmic inclusions and rimmed vacuoles are seen on mGT staining. (B) Reducing bodies are positive on melanodine–NBT staining. (C) Diffuse strong immunoreactivity to FHL1 is seen in both MyHC-slow positive and negative fibers (D). Bar = 20 μm.

severe weakness and absence of early contractures as seen in our patient and his brother.

Indeed the presence of RBs in RBM, and the retrospective identification of RBs in RSS patient reported here and SPM patient (unpublished data) suggests that *FHL1* is the causative gene for a variety of clinical disorders with RBs as the common diagnostic pathological finding. On the basis of our results, *FHL1* can be one of the causative genes for RSS.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Exploratory Research from Japan Society for the Promotion of Science; by "Research on Psychiatric and Neurological Diseases and Mental Health" of "Health Labour Sciences Research Grant" and the "Research Grant for Nervous and Mental Disorders" from the Ministry of Health, Labour, and Welfare; by Research on Health Sciences focusing on Drug Innovation from the Japanese Health Sciences Foundation; and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

[1] Chu PH, Ruiz-Lozano P, Zhou Q, et al. Expression patterns of FHL/SLIM family members suggest important functional roles in skeletal muscle and cardiovascular system. Mech Dev 2000;95:259–65.
[2] Robinson PA, Brown S, McGrath MJ, et al. Skeletal muscle LIM protein 1 regulates integrin-mediated myoblast adhesion, spreading, and migration. Am J Physiol Cell Physiol 2003;284:C681–695.
[3] McGrath MJ, Cottle DL, Nguyen MA, et al. Four and a half LIM protein 1 binds myosin-binding protein C and regulates myosin filament formation and sarcomere assembly. J Biol Chem 2006;281:7666–83.
[4] Qin H, Wang J, Liang Y, et al. RING1 inhibits transactivation of RBP-J by Notch through interaction with LIM protein KyoT2. Nucleic Acids Res 2004;32:1492–501.
[5] Schessl J, Zou Y, McGrath MJ, et al. Proteomic identification of FHL1 as the protein mutated in human reducing body myopathy. J Clin Invest 2008.
[6] Quinzii CM, Vu TH, Min KC, et al. X-linked dominant scapuloperoneal myopathy is due to a mutation in the gene encoding four-and-a-half-LIM protein 1. Am J Hum Genet 2008;82:208–13.
[7] Windpassinger C, Schoser B, Straub V, et al. An X-linked myopathy with postural muscle atrophy and generalized hypertrophy, termed XMPMA, is caused by mutations in FHL1. Am J Hum Genet 2008;82:88–99.
[8] Goebel HH, Halbig LE, Goldfarb L, et al. Reducing body myopathy with cytoplasmic bodies and rigid spine syndrome: a mixed congenital myopathy. Neuropediatrics 2001;32:196–205.
[9] Wilhelmsen KC, Blake DM, Lynch T, et al. Chromosome 12-linked autosomal dominant scapuloperoneal muscular dystrophy. Ann Neurol 1996;39:507–20.
[10] Dubowitz V. Rigid spine syndrome: a muscle syndrome in search of a name. Proc R Soc Med 1973;66:219–20.

Circ J 2009; **73:** 158–161

Mutational Analysis of Fukutin Gene in Dilated Cardiomyopathy and Hypertrophic Cardiomyopathy

Takuro Arimura, DVM*; Yukiko K. Hayashi, MD**; Terumi Murakami, MD**; Yasushi Oya, MD†; Sayaka Funabe, MD††; Eri Arikawa-Hirasawa, MD††; Nobutaka Hattori, MD††; Ichizo Nishino, MD**; Akinori Kimura, MD*,‡

Background Mutations in *FKTN* encoding for fukutin cause Fukuyama-type congenital muscular dystrophy characterized by severe muscle wasting and hypotonia with mental retardation. Fukuyama-type congenital muscular dystrophy is a recessive genetic trait. *FKTN* mutations in patients with dilated cardiomyopathy (DCM) have been investigated by our research group. The patients showed hyper-CKemia with mild or no muscle weakness and without mental retardation, suggesting that the clinical spectrum of *FKTN* mutations are wider than previously thought. The current study was designed to further explore the association of *FKTN* mutations with DCM or hypertrophic cardiomyopathy (HCM).

Methods and Results A total of 172 patients with DCM, 144 patients with familial HCM and 384 control individuals were analyzed for *FKTN* mutations. There was a DCM patient who was a compound heterozygote of a 3-kb insertion mutation and a missense mutation Cys101Phe. The patient showed hyper-CKemia with mild muscle involvement and no brain involvement. In contrast, 2 other DCM patients and 3 controls were heterozygous for the insertion mutation and normal allele, showing that the heterozygous insertion mutation itself was not associated with DCM. No mutation was found in the HCM patients.

Conclusions These observations indicated that the compound heterozygous *FKTN* mutation was a rare cause of DCM. Hyper-CKemia might be indicative of *FKTN* mutation in DCM. (*Circ J* 2009; **73:** 158–161)

Key Words: Cardiomyopathy; Genes; Genetics; Muscles

Idiopathic cardiomyopathy (ICM), which is mainly classified into 2 clinical phenotypes; hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM), is a primary heart muscle disorder caused by functional abnormalities in the cardiomyocytes and a major cause of sudden cardiac death and progressive heart failure[1] Although the etiology of ICM has not been completely elucidated, recent molecular genetic studies have shown that ICM can be caused by a variety of genetic abnormalities[1] Inheritance of familial HCM is usually autosomal dominant, whereas that of familial DCM is autosomal dominant, autosomal recessive, or X-linked recessive, ie, various type of disease inheritance can be found in DCM cases[2,3] It also should be noted that causative gene mutations could be found not only in familial cases but also in sporadic cases, indicating that the absence of family history cannot exclude a possibility of causative gene mutation in ICM cases[3] In addition, mutations in muscular dystrophy-causing genes might also lead to ICM phenotype, as exemplified that titin/connectin gene (*TTN*) mutations were found in patients with HCM[4] DCM[5] or tibial musclular dystrophy and limb-girdle type muscular dystrophy (LGMD)[6] and that Tcap gene (*TCAP*) mutations were found in HCM and DCM[7] as well as in LGMD[8] These observations indicate that there is an etiological overlap between ICM, and the skeletal muscle disorders[9]

Mutations in *FKTN* encoding for fukutin cause Fukuyama-type congenital muscular dystrophy (FCMD; MIM253800), the second most common muscular dystrophy in Japan after Duchenne muscular dystrophy. FCMD is an autosomal recessive disease manifested with severe muscle wasting and mental retardation[10,11] The majority of the FCMD patients were homozygous for a 3-kb insertion in the 3' non-coding region of *FKTN*, whereas a small population of FCMD patients were compound heterozygotes of the 3-kb insertion and a missense mutation[12,13] In addition, we recently identified compound heterozygotes of the insertion and a missense mutation in 2 sibling cases and 2 sporadic cases of DCM, who manifested with minimal muscle weakness and elevated serum creatine kinase (CK) concentration, hyper-CKemia, but not mental retardation[14] However, it remains unknown whether *FKTN* mutation can be associated with ICM not accompanied by signs of muscular dystrophy and in which type of ICM patients who should be examined for *FKTN* mutations as a disease-causing gene.

In the present study, we searched for *FKTN* mutations in a large panel of patients with DCM or HCM. We found a compound heterozygote of *FKTN* mutations in 1 out of 172 DCM patients, who also had mild muscular dystrophy and hyper-CKemia.

(Received July 24, 2008; revised manuscript received August 12, 2008; accepted August 21, 2008; released online November 17, 2008)

**Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, **Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), †Department of Neurology, National Center Hospital of Neurology and Psychiatry, NCNP, ††Department of Neurology, Juntendo University School of Medicine and ‡Laboratory of Genome Diversity, School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan*

Mailing address: Akinori Kimura, MD, Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. E-mail: akitis@mri.tmd.ac.jp

Methods

Study Population

We studied 172 genetically unrelated Japanese patients with DCM and 144 patients with familial HCM. Among the DCM patents, family history was not found in 100 patients (sporadic cases), whereas apparent family history was found in 72 patients; 4 were probands of sibling cases (possible autosomal recessive cases) and 68 patients were probands of DCM families, in which disease was inherited as a autosomal dominant genetic trait. In addition, family history consistent with autosomal dominant inheritance was found in all HCM patients. The patients were diagnosed based on medical history, physical examination, 12-lead electrocardiogram (ECG), echocardiography, and other special tests if necessary. Diagnostic criteria for DCM and HCM were described previously[7,15] These patients had been investigated for mutations in the known disease genes for ICM, such as sarcomere genes and Z-disc component genes[3] and no disease-causing mutations were identified. All patients showed no sign of brain involvement, ie, typical FCMD cases were clinically excluded. Control subjects were 384 unrelated healthy Japanese individuals selected at random. After acquiring informed consent, blood samples were obtained from each participant. The research protocol was approved by the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University and that of National Institute of Neuroscience, National Center of Neurology and Psychiatry.

Mutational Analysis of FKTN in ICM

Genomic DNA extracted from peripheral blood was subjected to polymerase chain reaction (PCR). To detect the 3-kb insertion in *FKTN*, we carried out PCR in all participants using 2 primer sets as described previously[14,16] Entire exons and their flanking regions of *FKTN* were directly sequenced on both strands by using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems Foster City, CA, USA) as reported previously[14]

Immunohistochemical Analysis

Monoclonal anti-α-DG (VIA4-1, Upstate Biotechnology, Lake Placid, NY, USA) and monoclonal anti-β-DG (43DAG1/8D5, Novocastra Laboratories, Newcastle upon Tyne, UK) were used for immunostaining of biopsied skeletal muscle samples as described previously[14]

Results

The 3-kb insertion mutation was searched in 172 DCM patients and 144 HCM patients. We found that 3 patients (all were sporadic DCM cases) carried the insertion mutation in the heterozygous state. This 3-kb insertion was not detected in other patients, but was identified in 3 out of 384 controls. We then sequenced all exons and adjacent introns of *FKTN* in the 3 sporadic DCM patients carrying the 3-kb insertion (**Fig 1A**) and found a missense mutation (c.302G>T, p.Cys101Phe) in one case (**Fig 1B**), suggesting that this patient was a compound heterozygote of *FKTN* mutations.

The patient was a 19-year-old female who manifested with exertional dyspnea and mild muscular weakness at neck and proximal extremities along with bilateral calf hypertrophy. She had shown hyper-CKemia (6,570 IU/L) without any muscle symptoms from the age of 17 years. Since then, she was followed up by physicians as a result of

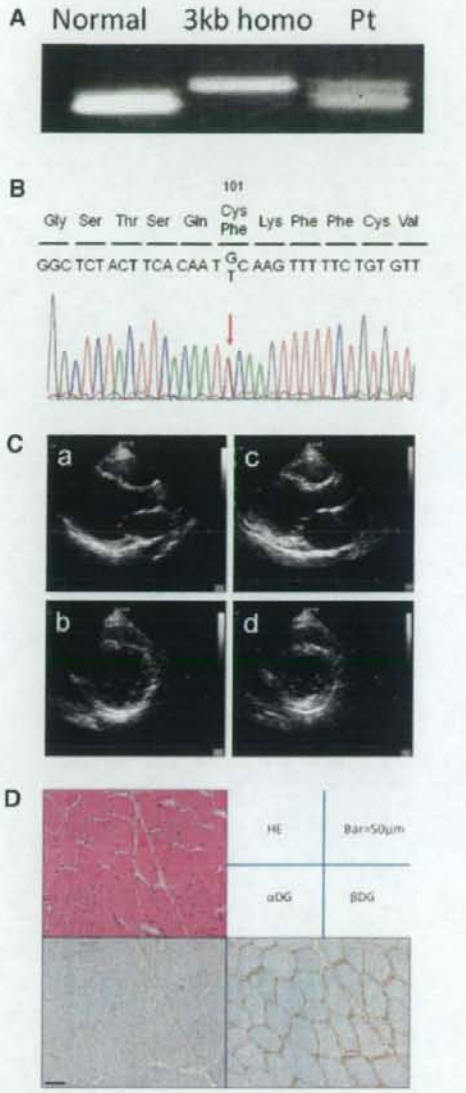

Fig 1. Gene and histochemical analyses of the dilated cardiomyopathy (DCM) patient carrying the *FKTN* mutation. (**A**) Detection of 3-kb insertion. Left, normal individual without mutation; middle, Fukuyama-type congenital muscular dystrophy patient carrying homozygous 3-kb insertion; right, the DCM patient with *FKTN* mutation. The patient showed both normal and insertion bands. (**B**) Direct sequencing data from the DCM patient. Polymerase chain reaction products containing exon 4 of *FKTN* gene from the patient were directly sequenced. Nucleotide sequences are shown along with predicted amino acid sequences. An arrowhead indicates the mutation resulting in TGC (Cys) to TTC (Phe) change. (**C**) Echocardiography of the patient. Endodiastolic (**a, b**) and endosystolic (**c, d**) data for sagital (**a, c**) and vertical (**b, d**) views showing left ventricular dilation. (**D**) Hematoxylin and eosin staining (HE) and immunohistochemical analysis. On HE, only mild variation in fiber size was found. Immunohistochemical analysis using a monoclonal antibody VIA4-1 that recognizes heavily-glycosylated form of α-dystroglycan (αDG), showed reduced sarcolemmal staining, whereas the staining of β-dystroglycan (βDG) using monoclonal antibody 43DAG1/8D5 showed no abnormality. Bar=50 μm.

the hyper-CKemia of unknown etiology. Diffuse left ventricular hypokinesis with left ventricular ejection fraction (LVEF) of 38% was observed at the age of 18 years, along with diffuse muscle atrophy and mild necrosis-regeneration process in biceps brachii muscle biopsy. She felt excertional dyspnea from the age of 18 years and when she was 19 years old, her ECG showed incomplete right bundle branch block, and her echocardiogram showed systolic dysfunction with ventricular dilatation (LVEF, 41%; left ventricular end-diastolic diameter, 53 mm; left ventricular end-systolic diameter, 43 mm; fractional shortening, 20%), whereas no ventricular hypertrophy was observed (inter ventricular septum, 6 mm; posterior wall, 7 mm). Biochemical analysis showed that she had hyper-CKemia (2,485 IU/L). Immunohistochemical analysis of biopsied muscle sample showed marked decrease of α-dystroglycan staining, whereas distribution and expression of β-dystroglycan was not changed (**Fig 1B**). This finding was consistent with *FKTN* mutations;[14] albeit that no family history of DCM or muscle disease was evident with her. From these observations, she was finally diagnosed as LGMD manifested with mild DCM phenotype.

In addition, we sequenced the entire coding regions and adjacent introns of *FKTN* from 72 patients with familial DCM (4 consistent with recessive inheritance and 68 with dominant inheritance). The sequencing analyses showed 2 variations, 1 non-synonymous change in exon 5 (c.608G>A, p.Arg203Gln) and 1 synonymous change in exon 8 (c.1026C>A, p.Leu342Leu), in several patients. However, both variations were reported to be polymorphisms in the SNP database (rs34787999 and rs17309806, respectively), suggesting that these were polymorphisms not related with DCM.

Discussion

The 3-kb insertion into the 3'-untranslated region of the *FKTN*, which has been derived from a single ancestral founder and causes a significant reduction of *FKTN* mRNA, could cause FCMD in homozygous states or in compound heterozygous states with another point mutation.[12,13] FCMD is one of the most severe congenital muscular dystrophy in combination with brain malformation, principally cerebral and cerebellar cortical dysplasia.[10,11] In contrast to the severely affected skeletal muscle, cardiac muscle involvement is quite rare in FCMD patients. However, we recently showed that the compound heterozygous mutations could also be associated with DCM accompanied by minimal limb girdle muscle involvement and normal intelligence.[14] These observations implied the wide phenotypic spectrum of the *FKTN* mutations. In the current study, we identified a patient carrying the 3-kb insertion and a missense mutation, who manifested with DCM and mild skeletal muscle phenotype. Clinical phenotype of the patient in this study was similar to those reported previously;[14] further supporting that the compound heterozygous mutation was associated with DCM. Because we have not examined her parents for the *FKTN* mutations, we could not formally exclude a possibility that these 2 mutations were in *trans* and not in *cis*. However, if the mutations were in *cis*, this patient should have one normal allele and the other non-expressing allele due to the 3-kb insertion, which is in a similar situation as the heterozygote of the 3-kb mutation; the situation not causing any disease phenotypes as discussed below.

The 3-kb insertion was also found in 2 other sporadic DCM cases, but these patients did not carry any additional *FKTN* mutations nor did they show hyper-CKemia, indicating that heterozygote of the insertion mutation and normal allele did not manifest with cardiomyopathy or muscle diseases. In addition, we identified 3 heterozygous carriers of the 3-kb insertion in 384 Japanese controls (0.78%), and this carrier frequency was similar to those previously reported by 2 other groups (6 in 676; 0.89%[16] and 15 in 2,814; 0.53%[17]). In this study, we investigated familial HCM patients for *FKTN* mutations even though the disease was inherited as an autosomal dominant trait as in the most cases of familial DCM. Because mutations in the muscular dystrophy genes such as *TTN* and *TCAP* cause skeletal muscle disease as the autosomal recessive trait and cardiomyopathy (HCM or DCM) as the autosomal dominant trait, we had not been able to exclude a possibility of *FKTN* mutations in autosomal dominant cases. However, no *FKTN* mutation was found in the patients with familial HCM as in familial DCM, examined in this study. These observations suggest that *FKTN* mutations should be considered as a cause of DCM, albeit not a major cause, especially in the sporadic cases or sibling cases.

What was the characteristic feature of DCM caused by *FKTN* mutations? The patient carrying the causative *FKTN* mutations showed hyper-CKemia before manifesting with cardiomyopathy and skeletal muscle symptoms. All the patients carrying the compound heterozygous mutations in the previous study had elevated serum CK concentrations, although they showed no or minimal skeletal muscle phenotypes.[14] The hyper-CKemia can also be found in the patients carrying *FKTN* mutations affected with FCMD[18] or LGMD.[19,20] These observations are in good agreement with the association between the *FKTN* mutations and hyper-CKemia. In our cohort of DCM patients, we identified disease-causing mutations in 4 sporadic DCM patients who showed continuously hyper-CKemia. One was the patient carrying the *FKTN* mutations reported here, whereas the other 3 patients had abnormalities in the dystrophin gene (DMD) with a deletion of exon 3, exon 44, or exons 45–51. The DCM patients with DMD mutations showed elevated serum CK concentrations of approximately 500–1,000 IU/L. The finding was in part consistent with that DCM patients carrying DMD mutations were reported to show hyper-CKemia even though they had no or minimal symptoms of muscle involvement.[21,22] These observations suggested that hyper-CKemia in patients with DCM might be an indicative sign of *FKTN* or *DMD* mutations.

In summary, we have investigated *FKTN* mutations in a large panel of patients with DCM or HCM and found that a sporadic DCM case with hyper-CKemia was a compound heterozygote of *FKTN* mutations.

Acknowledgements

We are grateful to Drs H. Toshima, H. Nishi, K. Matsuyama, H. Kagiyama, T. Sakamoto, K. Kawai, K. Kawamura, R. Kusukawa, M. Nagano, Y. Nimura, R. Okada, T. Sugimoto, H. Tanaka, H. Yasuda, F. Numano, K. Fukuda, S. Ogawa, A. Matsumori, S. Sasayama, R. Nagai, and Y. Yazaki for their contributions in clinical evaluation and blood sampling from patients with DCM. We also thank Dr M. Yanokura, Ms M. Emura and Ms A. Nishimura for their technical assistance. This work was supported in part by Grant-in-aids from the Ministry of Education, Culture, Sports, Science and Technology, Japan; Grant-in-Aids for Scientific Research from the Japan Society for the Promotion of Science, Research on Psychiatric and Neurological Diseases and Mental Health from Health and Labor Sciences Research Grants, and research grants for Idiopathic Cardiomyopathy and for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare, Japan, Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical

Innovation, and Association Française contre les Myopathies.

References

1. Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, et al. Contemporary definitions and classification of the cardiomyopathies: An American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. *Circulation* 2006; **113:** 1807–1816.
2. Michels VV, Moll PP, Miller FA, Tajik AJ, Chu JS, Driscoll DJ, et al. The frequency of familial dilated cardiomyopathy in a series of patients with idiopathic dilated cardiomyopathy. *N Engl J Med* 1992; **326:** 77–82.
3. Kimura A. Molecular etiology and pathogenesis of hereditary cardiomyopathy. *Circ J* 2008; **72**(Suppl)**:** A-38–A-48.
4. Satoh M, Takahashi M, Sakamoto T, Hiroe M, Marumo F, Kimura A, et al. Structural analysis of the titin gene in hypertrophic cardiomyopathy: Identification of a novel disease gene. *Biochem Biophys Res Commun* 1999; **262:** 411–417.
5. Itoh-Satoh M, Hayashi T, Nishi H, Koga Y, Arimura T, Koyanagi T, et al. Titin mutations as the molecular basis for dilated cardiomyopathy. *Biochem Biophys Res Commun* 2002; **291:** 385–393.
6. Hackman P, Vihola A, Haravuori H, Marchand S, Sarparanta J, De Seze J, et al. Tibial muscular dystrophy is a titinopathy caused by mutations in TTN, the gene encoding the giant skeletal-muscle protein titin. *Am J Hum Genet* 2002; **71:** 492–500.
7. Hayashi T, Arimura T, Itoh-Satoh M, Ueda K, Hohda S, Inagaki N, et al. Tcap gene mutations in hypertrophic cardiomyopathy and dilated cardiomyopathy. *J Am Coll Cardiol* 2004; **44:** 2192–2201.
8. Moreira ES, Wiltshire TJ, Faulkner G, Nilforoushan A, Vainzof M, Suzuki OT, et al. Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. *Nature Genet* 2000; **24:** 163–166.
9. McNally E, Allikian M, Wheeler MT, Mislow JM, Heydemann A. Cytoskeletal defects in cardiomyopathy. *J Mol Cell Cardiol* 2003; **35:** 231–241.
10. Fukuyama Y, Osawa M, Suzuki H. Congenital progressive muscular dystrophy of the Fukuyama type–clinical, genetic and pathological considerations. *Brain Dev* 1981; **3:** 1–29.
11. Kondo-Iida E, Saito K, Tanaka H, Tsuji S, Ishihara T, Osawa M, et al. Molecular genetic evidence of clinical heterogeneity in Fukuyama-type congenital muscular dystrophy. *Hum Genet* 1997; **99:** 427–432.
12. Kobayashi K, Nakahori Y, Miyake M, Matsumura K, Kondo-Iida E, Nomura Y, et al. An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature* 1998; **394:** 388–392.
13. Kondo-Iida E, Kobayashi K, Watanabe M, Sasaki J, Kumagai T, Koide H, et al. Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD). *Hum Mol Genet* 1999; **8:** 2303–2309.
14. Murakami T, Hayashi YK, Noguchi S, Ogawa M, Nonaka I, Tanabe Y, et al. Fukutin gene mutations cause dilated cardiomyopathy with minimal muscle weakness. *Ann Neurol* 2006; **60:** 597–602.
15. Arimura T, Hayashi T, Terada H, Lee SY, Zhou Q, Takahashi M, et al. A Cypher/ZASP mutation associated with dilated cardiomyopathy alters the binding affinity to protein kinase C. *J Biol Chem* 2004; **279:** 6746–6752.
16. Kato R, Kawamura J, Sugawara H, Niikawa N, Matsumoto N. A rapid diagnostic method for a retrotransposal insertional mutation into the FCMD gene in Japanese patients with Fukuyama congenital muscular dystrophy. *Am J Med Genet A* 2004; **127:** 54–57.
17. Watanabe M, Kobayashi K, Jin F, Park KS, Yamada T, Tokunaga K, et al. Founder SVA retrotransposal insertion in Fukuyama-type congenital muscular dystrophy and its origin in Japanese and Northeast Asian populations. *Am J Med Genet A* 2005; **138:** 344–348.
18. Silan F, Yoshioka M, Kobayashi K, Simsek E, Tunc M, Alper M, et al. A new mutation of the fukutin gene in a non-Japanese patient. *Ann Neurol* 2003; **53:** 392–396.
19. Godfrey C, Escolar D, Brockington M, Clement EM, Mein R, Jimenez-Mallebrera C, et al. Fukutin gene mutations in steroid-responsive limb girdle muscular dystrophy. *Ann Neurol* 2006; **60:** 603–610.
20. Lin YC, Murakami T, Hayashi YK, Nishino I, Nonaka I, Yuo CY, et al. A novel FKRP gene mutation in a Taiwanese patient with limb-girdle muscular dystrophy 2I. *Brain Dev* 2007; **29:** 234–238.
21. Mirabella M, Servidei S, Manfredi G, Ricci E, Frustaci A, Bertini E, et al. Cardiomyopathy may be the only clinical manifestation in female carriers of Duchenne muscular dystrophy. *Neurology* 1993; **43:** 2342–2345.
22. Feng J, Yan J, Buzin CH, Towbin JA, Sommer SS. Mutations in the dystrophin gene are associated with sporadic dilated cardiomyopathy. *Mol Genet Metab* 2002; **77:** 119–126.

Neuromuscular Disorders 19 (2009) 29–36

Contents lists available at ScienceDirect

Neuromuscular Disorders

journal homepage: www.elsevier.com/locate/nmd

Nuclear changes in skeletal muscle extend to satellite cells in autosomal dominant Emery-Dreifuss muscular dystrophy/limb-girdle muscular dystrophy 1B

Young-Eun Park [a], Yukiko K. Hayashi [a,*], Kanako Goto [a], Hirofumi Komaki [b], Yuichi Hayashi [c], Takashi Inuzuka [c], Satoru Noguchi [a], Ikuya Nonaka [a], Ichizo Nishino [a]

[a] Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, 187-8502 Tokyo, Japan
[b] Department of Child Neurology, National Center Hospital of Neurology and Psychiatry, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, 187-8551 Tokyo, Japan
[c] Department of Neurology and Geriatrics, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

ARTICLE INFO

Article history:
Received 22 May 2008
Received in revised form 24 September 2008
Accepted 26 September 2008

Keywords:
Autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD)
Limb girdle muscular dystrophy type 1B (LGMD1B)
A-type lamins
Nucleus
Satellite cell
Pax7
MyoD
Regeneration

ABSTRACT

Autosomal forms of Emery-Dreifuss muscular dystrophy (AD-/AR-EDMD) and limb-girdle muscular dystrophy type 1B (LGMD1B) are caused by mutations in the gene encoding A-type lamins (*LMNA*). A-type lamins are major components of nuclear lamina and known to have important roles in maintaining nuclear integrity. *LMNA* mutations are also suggested to cause reduced myogenic differentiation potentials, implying that satellite cell nuclei in AD-EDMD/LGMD1B are likewise affected. We examined nuclear changes of skeletal muscles including satellite cells from four patients with AD-EDMD/LGMD1B by light and electron microscopy. We found that 92.5 ± 5.0% of myonuclei had structural abnormalities, including shape irregularity and/or chromatin disorganization, and the presence of peri-/intranuclear vacuoles. Chromatin changes were also observed in 50% of the satellite cell nuclei. Increased number of Pax7-positive nuclei, but fewer number of MyoD-positive nuclei were seen on immunohistochemical analyses, suggesting functional alteration of satellite cells in addition to the nuclear morphological changes in AD-EDMD/LGMD1B.

1. Introduction

Autosomal dominant and recessive forms of Emery-Dreifuss muscular dystrophy (AD- and AR-EDMD, respectively) are caused by the mutations in the *LMNA* gene that encodes A-type lamins, and are clinically characterized by muscle weakness with humeroperoneal distribution, early onset joint contractures and dilated cardiomyopathy with conduction defects [1]. *LMNA* mutations can also cause limb-girdle muscular dystrophy type 1B (LGMD1B), which has the clinical feature of proximal dominant muscle weakness with cardiac involvement [2].

Lamins form the nuclear lamina meshwork at the inner nuclear membrane and are suggested to have an important role in the maintenance of nuclear architecture. *LMNA* mutations or *Lmna* knockout are proven to cause nuclear dysmorphism and increase the fragility against mechanical stress in cultured cell analyses [3,4], supporting the notion that the nuclear lamina holds nuclear integrity. From this concept, myonuclei are presumably affected when *LMNA* is mutated since the skeletal muscle is frequently exposed to strong mechanical stress through the process of muscle contraction and relaxation. *LMNA* mutations were also shown to cause altered differentiation potential and kinetics of skeletal muscle cells by *in vitro* cell analyses [5].

In this study, we examined the morphological changes of myonuclei, and also nuclei of satellite cells in the skeletal muscles from four patients with genetically confirmed AD-EDMD/LGMD1B by using light and electron microscope. Further, we also counted the number of the myonuclei expressing Pax7 and MyoD to evaluate the differentiation potential of satellite cells in AD-EDMD/LGMD1B.

2. Patients and methods

2.1. Patients

All clinical materials used in this study were obtained for the diagnostic purposes with informed consent. Clinical information of four patients with AD-EDMD/LGMD1B is summarized in Table 1. In brief;

* Corresponding author. Tel.: +81 42 341 2711; fax: +81 42 346 1742.
E-mail address: hayasi y@ncnp.go.jp (Y.K. Hayashi).

0960-8966/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.nmd.2008.09.018

Patient 1 is a 4-year and 5-month-old girl who was clinically suspected to have LGMD. She presented with lordosis and waddling gait from the age of 2 years. On physical examination, she showed proximal limb muscle weakness with wasting and calf hypertrophy. Serum creatine kinase (CK) level was elevated to 1408 IU/L (normal: 51–197 IU/L). Sequence analysis of *LMNA* revealed a heterozygous mutation of c.1357C > T (p.R453W) in exon 7.

Patient 2 is a 5-year and 8-month-old girl, who was noticed to have limited ankle flexion and elevation of serum CK level at the age of 2 years. On admission, she showed generalized muscle wasting, lordosis, and ankle/knee joint contractures. Serum CK levels were elevated from 900 to 1500 IU/L. The presence of a heterozygous mutation of c.746G > A (p.R249Q) in exon 4 of *LMNA* confirmed the diagnosis of EDMD.

Patient 3 is an 8-year and 1-month-old girl who had muscle weakness and delayed motor milestones. She walked without support at the age of 1 year and 4 months, but gradually lost independent ambulation at around 3 years of age. Scoliosis was noted on physical examination. Serum CK level was 351 IU/L. Echocardiography showed mildly decreased cardiac motility. Genetic analysis revealed a heterozygous missense mutation of c.875T > C (p.L292P) in exon 5 of *LMNA*.

Table 1
Clinical and genetic information of four patients with AD-EDMD/LGMD1B.

	Patient 1	Patient 2	Patient 3	Patient 4
Age/Sex	4 years/F	5 years/F	8 years/F	52 years/F
Age at onset	2 years	2 years	1 year 4 months	6 years
Skeletal muscle symptoms	Prox. limb weakness Lordosis Calf hypertrophy	Prox. limb weakness Lordosis	Prox. limb weakness Scoliosis Loss of ambulation	Prox. limb weakness Facial/neck weakness
Joint contracture	None	Ankles & knees	None	None
Cardiac symptoms	None	None	Wall motion decrease on echocardiography	Sick sinus syndrome + ventricular tachycardia → pacemaker insertion
Serum CK (IU/L)	1408	900–1500	351	117
LMNA mutation	p.R453W	p.R249Q	p.L292P	p.D511fs

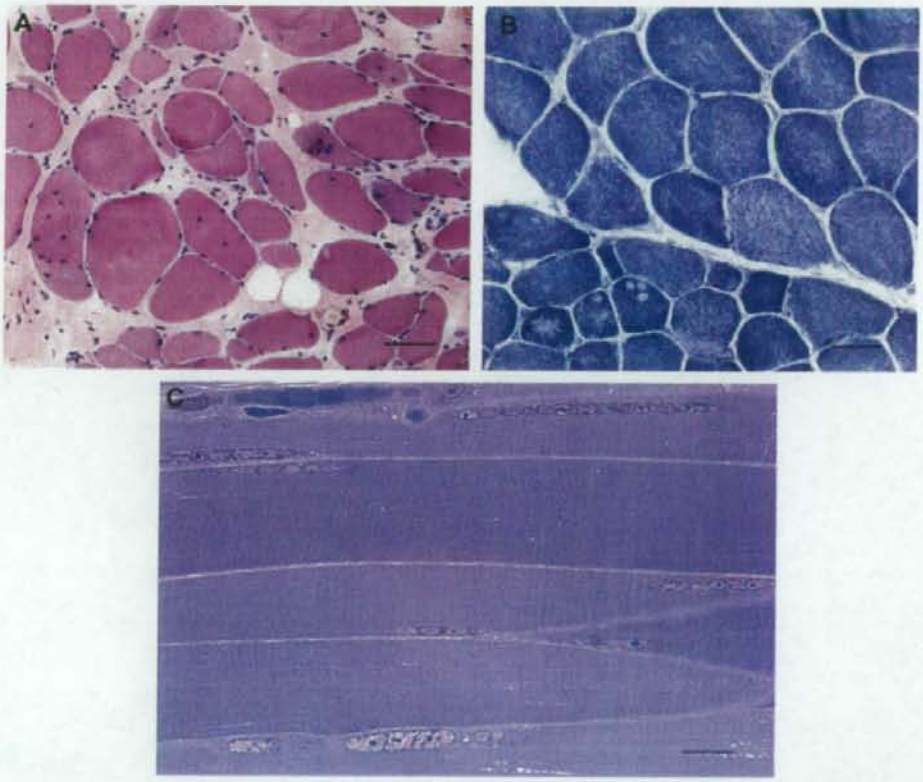

Fig. 1. Light microscopic observation of skeletal muscle. (A) H&E staining in patient 3 shows that the number of nuclei within one fiber is markedly increased. (B) NADH staining in patient 4 shows well-preserved myofibril organization in many fibers, whereas a few fibers have core/mini core-like structure. (C) Semi-thin section in patient 1 stained by toluidine blue contains nuclear chains of variable size in subsarcolemmal regions. Bar = 100 μm (A and B), 50 μm (C).

Patient 4 is a 52-year-old female, whose family history was suggestive of an autosomal dominant inheritance because her mother and her mother's sister had muscle weakness and cardiac enlargement/arrhythmia. She had been a slow runner since childhood and waddling gait was noticed at the age of 6 years. She felt difficulty in climbing stairs from 35 years of age. A diagnosis of sick sinus syndrome with ventricular tachycardia was given at 46 years whereby a pacemaker was inserted. Serum CK level was 117 IU/L. Mutation analysis showed a novel heterozygous frame-shift mutation of c.1527-1529TAC < AA (p.D511fs) in exon 9 of *LMNA*.

For comparison of histochemical and immunohistochemical analyses, muscle specimen from age-matched patients with Duchenne/Becker muscular dystrophy (DMD/BMD) ($n = 4$) and normal controls ($n = 4$) were also analyzed.

2.2. Histochemical analyses

Biopsied muscle specimens were flash-frozen in isopentane chilled with liquid nitrogen. Serial 10 μm-thick frozen sections were analyzed with a set of histochemical staining including hematoxylin and eosin (H&E), modified Gomori trichrome, nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome *c* oxidase and myosin ATPase. On ATPase staining, the ratio of type 2C fibers was considered in the patients with AD-EDMD/LGMD1B, and age-matched Duchenne/Becker muscular dystrophy (DMD/BMD) and normal controls.

2.3. Immunohistochemical analysis

Serial 6 μm-thick frozen muscle sections were fixed with acetone. Immunostaining was performed using standard method. To exclude other diagnosable muscular dystrophies, we used antibodies against dystrophin (Dys1, Dys2, and Dys3 from Novocastra Laboratories, Newcastle upon Tyne, UK), α-dystroglycan (Upstate Biotechnology, Lake Placid, NY), β-dystroglycan (Novocastra Laboratories), dysferlin (Novocastra Laboratories), α-, β-, γ-, δ-sarcoglycan (Novocastra Laboratories), caveolin-3 (Novocastra Laboratories), laminin-α2 chain (Chemicon, Temecula, CA), collagen VI (Abcam, Cambridge, UK), emerin (Novocastra Laboratories), and lamin A/C (Abcam, Tokyo, Japan).

For the satellite cell analyses, primary antibodies against Pax7 (Developmental Studies Hybridoma Bank, The University of Iowa, IA), MyoD (Santa Cruz Biotechnology, CA), and laminin α2 (Chemicon) were used. The stained sections were mounted together with DAPI for nuclear localization, and examined under immunofluorescence microscope (Carl Zeiss). In this study, immunopositive nuclei for Pax7 or MyoD located beneath the laminin α2-positive basal lamina are defined as Pax7-positive or MyoD-positive. From each specimen, 200–300 nuclei were recruited for analyses. The number of Pax7-positive or MyoD-positive nuclei was counted and compared with the total number of myonuclei to obtain the ratio of Pax7 and MyoD-positive nuclei per 100 myonuclei.

To detect DNA fragmentation, the TUNEL method was performed on frozen muscle specimens by using in situ apoptosis detection kit (Takara, Shiga, Japan) according to the manufacturer's instruction.

2.4. Electron microscopic observation

Muscle specimens were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After shaking with a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate, and 0.2 M s-collidine for 2–3 h, samples were embedded in epoxy resin. Semi-thin sections (1 μm-thickness) were stained with toluidine blue. Ultrathin sections of 50 nm thickness were stained with uranyl acetate and lead citrate, and then examined under H-600 transmission electron microscope (Hitachi, Japan) at 75 kV. We examined morphological changes of more than 100 myonuclei in each patient and 100 myonuclei in one DMD patient as a control. We also examined 20 satellite cell nuclei in each patient. Myonuclei in significantly degenerated myofibers were excluded from the count. We calculated the frequency of myonuclei showing markedly irregular membrane contours and nuclear chains. We also counted the number of nuclei having only thin heterochromatin (scanty heterochromatin) beneath the nuclear membrane. Mean percentage ± standard deviation (SD) of abnormal nuclei was calculated. To detect the euchromatin changes, intensities of photocopied euchromatin regions in randomly sampled 40 nuclei of each patient and a DMD control were measured by using Photoshop. We

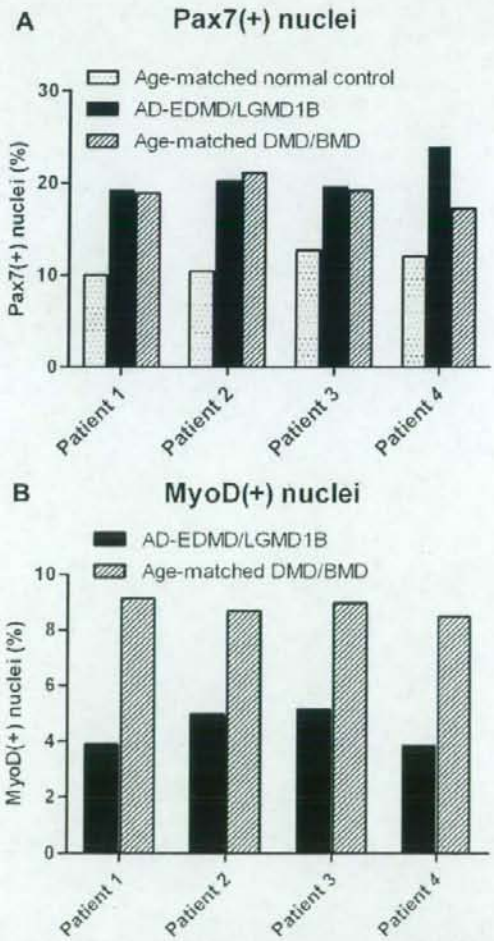

Fig. 2. Graphs showing the ratio of Pax7-positive (A) and MyoD-positive nuclei (B) in the patients with AD-EDMD/LGMD1B, and age-matched DMD/BMD and normal controls. (A) The ratio of Pax7-positive nuclei is increased both in AD-EDMD/LGMD1B and DMD/BMD patients compared with normal controls. (B) MyoD-positive nuclei in AD-EDMD/LGMD1B patients are much less than in DMD/BMD patients.