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Dystrophin deficiency in canine X-linked muscular dystrophy in Japan (CXMD_J) alters myosin heavy chain expression profiles in the diaphragm more markedly than in the tibialis cranialis muscle

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Abstract

Background: Skeletal muscles are composed of heterogeneous collections of muscle fiber types, the arrangement of which contributes to a variety of functional capabilities in many muscle types. Furthermore, skeletal muscles can adapt individual myofibers under various circumstances, such as disease and exercise, by changing fiber types. This study was performed to examine the influence of dystrophin deficiency on fiber type composition of skeletal muscles in canine X-linked muscular dystrophy in Japan (CXMD_J), a large animal model for Duchenne muscular dystrophy.

Methods: We used tibialis cranialis (TC) muscles and diaphragms of normal dogs and those with CXMD_J at various ages from 1 month to 3 years old. For classification of fiber types, muscle sections were immunostained with antibodies against fast, slow, or developmental myosin heavy chain (MHC), and the number and size of these fibers were analyzed. In addition, MHC isoforms were detected by gel electrophoresis.

Results: In comparison with TC muscles of CXMD_J, the number of fibers expressing slow MHC increased markedly and the number of fibers expressing fast MHC decreased with growth in the affected diaphragm. In populations of muscle fibers expressing fast and/or slow MHC(s) but not developmental MHC of CXMD_J muscles, slow MHC fibers were predominant in number and showed selective enlargement. Especially, in CXMD_J diaphragms, the proportions of slow MHC fibers were significantly larger in populations of myofibers with non-expression of developmental MHC. Analyses of MHC isoforms also indicated a marked increase of type I and decrease of type IIA isoforms in the affected diaphragm at ages over 6 months. In addition, expression of developmental (embryonic and/or neonatal) MHC decreased in the CXMD_J diaphragm in adults, in contrast to continuous high-level expression in affected TC muscle.

Conclusion: The CXMD_J diaphragm showed marked changes in fiber type composition unlike TC muscles, suggesting that the affected diaphragm may be effectively adapted toward dystrophic stress by switching to predominantly slow fibers. Furthermore, the MHC expression profile in the CXMD_J diaphragm was markedly different from that in *mdx* mice, indicating that the dystrophic dog is a more appropriate model than a murine one, to investigate the mechanisms of respiratory failure in DMD.

Background

Duchenne muscular dystrophy (DMD) is an X-linked, lethal disorder of skeletal muscle caused by mutations in the dystrophin gene, which encodes a large sub-sarcolemmal cytoskeletal protein, dystrophin. DMD is characterized by a high incidence (1 in 3,500 boys) and a high frequency of *de novo* mutation [1]. The absence of dystrophin is accompanied by the loss of dystrophin-associated glycoprotein complex from the sarcolemma, leading to reduce membrane stability of myofibers. This dysfunction results in progressive muscle weakness, cardiomyopathy, and subsequent early death by respiratory or heart failure in DMD patients.

For basic and therapeutic studies of DMD, it is very important to perform analysis and evaluation using dystrophin-deficient animal models, such as the *mdx* mouse and dystrophic dog. The *mdx* mouse has been well utilized in many DMD studies, but the murine model shows moderate dystrophic changes unlike severe human DMD [2]. In contrast, golden retriever muscular dystrophy (GRMD) shows similar dystrophic phenotypes to those of human patients: elevated serum CK level, gross muscle atrophy with joint contracture, cardiomyopathy, prominent muscle necrosis, degeneration with mineralization and concurrent regeneration, and endomysial and perimysial fibrosis [3]. Therefore, the dystrophic dog is more suitable than the *mdx* mouse for studies to gain insight into the pathogenic and molecular biological mechanisms of human DMD, as well as for pre-clinical trials [4]. Therefore, we have recently established a colony of beagle-based canine X-linked muscular dystrophy in Japan (CXMD_J) [5], and have demonstrated that CXMD_J also exhibited severe symptoms similar to GRMD. To date, we have utilized the littermates of the CXMD_J colony for pathological [6,7], molecular biological [8], and therapeutic examinations [9] of DMD.

Skeletal muscles are composed of heterogeneous populations of muscle fiber types, which contribute to a variety of functional capabilities. In addition, muscle fibers can adapt to diverse situations, such as aging, exercise, and muscular diseases, by changing fiber size or fiber type composition. Therefore, it is important to analyze fiber types to evaluate the condition of skeletal muscle with disease. Fiber types can be distinguished by biochemical, metabolic, morphological, and physiological properties. One of the most informative methods for identification of fiber types is detection of myosin heavy chain (MHC) [10,11]. Myofibers express various MHC isoforms containing slow (type I), fast (types IIA, IIX, IIB), embryonic, and neonatal forms. MHC expression, however, seems to differ between animal species and muscle types. Three MHC isoforms (types I, IIA, and IIX) have been identified in limb skeletal muscles of human and dog, while the

fourth isoform, MHC IIB, is abundantly present in small mammals including mouse [10,11]. In addition, expression profiles of MHCs in dystrophin-deficient muscles have been widely examined in limb skeletal muscles of DMD patients [12] and animal models, such as the *mdx* mouse [13] and GRMD [14], but it has not been fully analyzed in skeletal muscles of a canine model. Furthermore, expanded studies of the diaphragm were restricted to that of the *mdx* mouse [13,15]. Therefore, it is important to perform detailed evaluation of fiber types and fiber sizes in limb skeletal muscles and the diaphragm of CXMD_J to understand adaptations toward disease by changes in fiber type composition in the skeletal muscles of human DMD.

In this study, to investigate fiber types of myofibers in dystrophin-deficient skeletal muscles of dystrophic dogs, we evaluated the expression profiles of MHCs in tibialis cranialis (TC) muscles and diaphragms of CXMD_J at various ages, by immunohistochemical and electrophoretic techniques. Briefly, we detected myofibers expressing fast type, slow type, and/or developmental MHCs. In addition, the numbers of fast or slow MHC fibers and the size distribution of these myofibers were analyzed among populations of muscle fibers with or without developmental MHC. The composition of MHC isoforms was also examined in pairs of normal and affected dogs at various ages. This is the first report of evaluation of the detailed distribution of fiber types in TC muscles and diaphragms of dystrophic dogs.

Methods

Animals

Experimental dogs were wild-type and dystrophic littermates at ages from 1 month to 3 years, from the beagle-based CXMD_J breeding colony at National Center of Neurology and Psychiatry (Tokyo, Japan) [5,6]. Within a few days after birth, the genotypes (wild-type, carrier, or dystrophy) of the littermates were determined by a snapback method of single-strand conformation polymorphism (SSCP) analysis [16], and the phenotypes were also confirmed by measuring serum CK level [5]. All animals were cared for and treated in accordance with the guidelines approved by Ethics Committee for Treatment of Laboratory Animals at NCNP, where three fundamental principles (replacement, reduction, and refinement) were also considered. Adult control and CXMD_J dogs (10 months to 3 years) were analyzed in early experiments (three to six animals). Series consisting of a pair of a normal dog and an affected littermate at ages of 1, 2, 4, 6 months, or 1 year old were examined in subsequent experiments. TC muscles and diaphragms were removed from the dogs after necropsy, in which euthanasia was performed by exsanguination under anesthesia with isoflurane taken to prevent unnecessary pain. TC muscle was used as a

representative limb skeletal muscle, and it corresponds to the tibialis anterior muscle in mice and humans. The muscle blocks were divided into pieces and frozen immediately in isopentane pre-cooled with liquid nitrogen.

Histological and immunohistochemical analysis

Serial transverse cryosections (10 μ m thick) were stained with hematoxylin and eosin (H&E), and immunostained using anti-MHC antibodies. Immunohistochemistry was performed as described previously [17]. Cryosections were incubated with the following primary antibodies: mouse monoclonal antibodies against fast type MHC (NCL-MHCf; Novocastra), slow type MHC (NCL-MHCs), and developmental MHC (NCL-MHCd). The primary antibodies were detected using a Vectastain[®] ABC kit (Vector Laboratories) and then visualized with diaminobenzidine. Images were recorded using a microscope (Eclipse E600; Nikon) equipped with a CCD camera (HV-D28S; Hitachi), and fiber types of individual myofibers from 400 to 1200 per muscle were identified, based on serial sections immunostained with three types of MHC antibodies. Subsequently, the fiber number of each group was counted, and fiber sizes were also measured using Image-Pro Plus (Media Cybernetics). Furthermore, the differences in MHC expression between two groups (normal, dMHC (-) vs affected, dMHC (-); affected, dMHC (-) vs affected, dMHC (+)), between muscles (TC muscle vs diaphragm), or among ages (1, 2, 4, 6 months, and 1 year) were evaluated by Yates's chi-square test.

Myosin extraction and gel separation

Myosin was extracted on ice for 60 min from cryosections, as described previously [18,19]. MHC isoforms were separated on 8% SDS-polyacrylamide gels containing 30% glycerol, according to the methods described previously [19,20] with some modifications. Briefly, aliquots of 0.4 μ g of total protein were loaded in each well of mini-gels (Bio-Rad). Electrophoresis was carried out at 60 V at 5°C for 48 h using upper buffer containing additional 10 mM 2-mercaptoethanol. The gels were stained with silver, and the image was scanned and analyzed using NIH image.

Results

MHC expression in TC muscle and diaphragm of adult CXMD₁

To investigate the relationship between the pathology and fiber types in dystrophic skeletal muscles of CXMD₁, we first examined histological features and MHC expression in TC muscles and diaphragms of normal and affected dogs at adult stages (10 months to 3 years old) (Fig. 1). In H&E-stained sections, affected muscles exhibited some dystrophic characteristics, such as necrosis, regeneration, cellular infiltration, fibrosis, fiber splitting, and fiber size variation. Especially, clusters of infiltrating cells were

prominently observed in TC muscles, while endomysial fibrosis was predominant in diaphragms.

We next detected expression of fast and slow type MHCs for fiber type identification, and further examined developmental MHC, which means neonatal and/or embryonic MHC, as a marker of regenerating fibers (Fig. 1). In TC muscles and diaphragms of adult normal dogs, individual myofibers showed expression of either fast or slow type MHC. In affected TC muscles, the proportions of fast or slow MHC fibers were similar between normal and affected muscles. In addition, large numbers of developmental MHC-expressing fibers were observed in clusters, and many of these fibers co-expressed fast type MHC. In the affected diaphragms, the numbers of fast MHC fibers were much lower than in the normal counterparts, and slow type MHC was expressed in almost all fibers. Furthermore, the numbers of developmental MHC fibers were less than in affected TC muscle, and almost all of these fibers co-expressed slow type MHC, unlike TC muscle. These results indicated that the influences of dystrophin deficiency on MHC expression are significantly different between TC muscle and the diaphragm of CXMD₁, suggesting that the diaphragm would be more greatly influenced with regard to the composition of fiber types and muscle regeneration than TC muscle.

MHC expression and fiber size distribution

To further evaluate the size distribution of individual myofibers related to MHC expression, we measured transverse areas of all muscle fibers within one area in TC muscle or diaphragm of adult CXMD₁ (Fig. 2 and Table 1). We then analyzed three types of MHC-positive fibers (fast, slow, and hybrid) among populations of myofibers expressing fast and/or slow type MHC(s) together with or without developmental MHC, which were defined as regenerating or non-regenerating fibers, respectively. In non-regenerating fibers of affected TC muscle and diaphragm, the proportion of slow MHC fibers increased and these fibers showed a larger size distribution than those in the normal counterparts, indicating increased number and enlarged fiber size of slow fibers (Fig. 2B and Table 1). Interestingly, fast MHC fibers disappeared in the adult CXMD₁ diaphragm.

In regenerating fibers of both affected muscles, the distributions of all three populations shifted to smaller sizes than those in the normal counterparts, and a large number of hybrid fibers co-expressing fast and slow type MHCs were observed at a high rate (Fig. 2C and Table 1). In addition, fast MHC fibers were predominant in a regenerating population in TC muscle, while slow MHC fibers were predominant in the diaphragm except for hybrid fibers. These observations suggested that fast fibers could be more susceptible to dystrophic stress than slow fibers, and

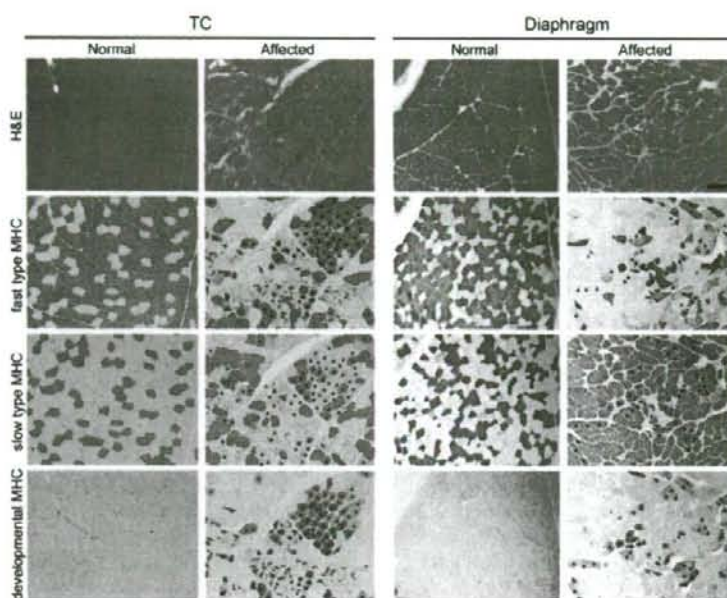


Figure 1
Representative images of histology (H&E) and expression of fast type, slow type, or developmental myosin heavy chain (MHC) in tibialis cranialis (TC) muscle and diaphragm of a normal (10 months old) or a CXMD₁ dog (11 months old). Identical parts of serial cross-sections are shown in longitudinal panels. In panels of affected muscles, dots show the fibers expressing developmental MHC. Bar: 200 μ m.

alteration of MHC expression and regeneration of muscle fibers would be different between TC muscle and the diaphragm.

Time courses of histology and MHC expression

To investigate how MHC expression alters together with growth of CXMD₁, we examined MHC expression in TC muscles and diaphragms of a normal or an affected littermate at various ages from neonatal to adult stages (1 month to 1 year old) in relation to histopathological features. Affected TC muscles showed mild lesions at 1 and 2 months old, but severe degenerative lesions were evident at over 4 months old (Fig. 3). Expression of fast or slow type MHC did not alter much with aging, and developmental MHC was expressed continuously (Fig. 4). In contrast, degenerative lesions were severe in the affected diaphragm at all ages examined (from 1 month old onward), and endomysial fibrosis was dominantly present over 6 months old (Fig. 3). Fast MHC fiber number decreased markedly, while the number of slow MHC fibers increased significantly in affected diaphragms after 6 months old (Fig. 5). In addition, expression of developmental MHC decreased at 6 months and 1 year

old. These observations indicated that MHC expression is altered greatly in the affected diaphragms after 6 months old, unlike TC muscles.

For quantitative evaluation of MHC expression in individual myofibers, we counted three types of MHC-expressing fibers among non-regenerating or regenerating populations within an area in the TC muscle or diaphragm of a normal or an affected littermate (Fig. 6). As normal muscles still expressed developmental MHC at 1 month old (Fig. 4 and 5), we performed the examinations at both adolescent (2 and 4 months old) and adult stages (10 or 11 months old). In normal dogs, the number of fast MHC fibers in TC muscle was three times greater than that of slow MHC fibers throughout aging, while the proportions in the diaphragms remained constant and equivalent between the two types (Fig. 6A). In non-regenerating fibers, the proportions of fiber types were not constant in affected TC muscles at the ages examined, but the majority of these fibers consisted of slow MHC fibers in the affected diaphragms (Fig. 6B). These observations indicated that slow fibers were already predominant in non-regenerating populations of CXMD₁ diaphragms at younger ages. In

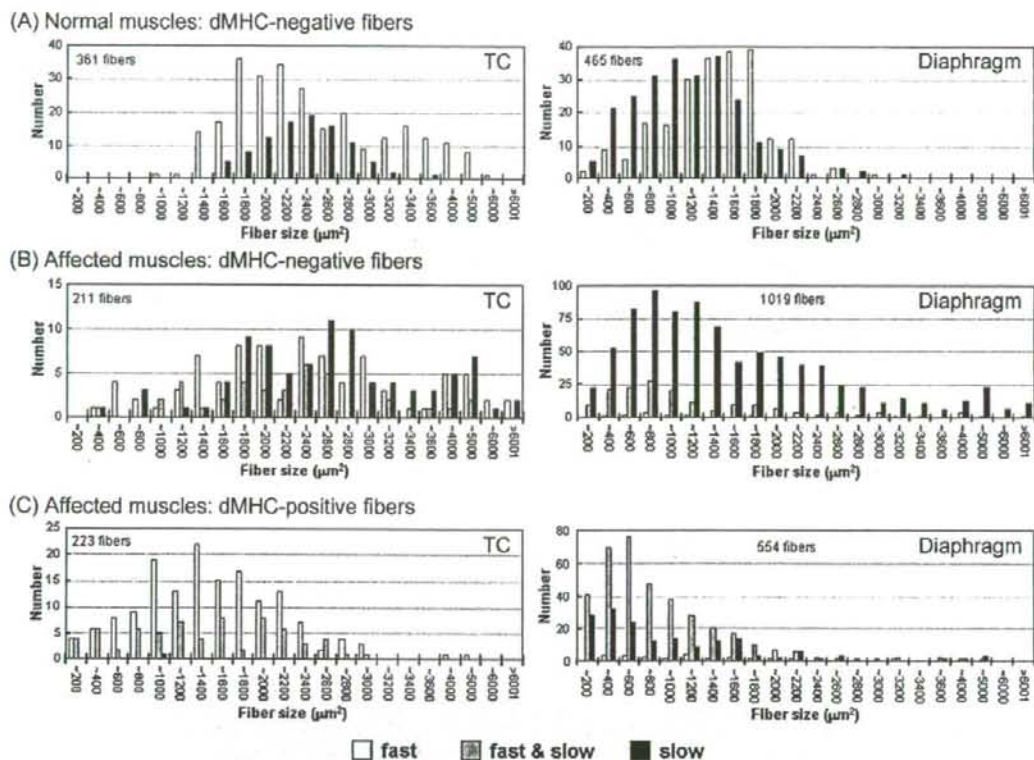


Figure 2

The size distribution of myofibers expressing fast and/or slow type MHCs in skeletal muscles of a normal (10 months old) or a CXMD₁ dog (11 months old). On the basis of expression of fast and slow type MHCs, all fibers within an area of TC muscle or diaphragm of a normal (A) or an affected dog (B, C) were classified into three types of MHC-positive fiber. Furthermore, fast (white), hybrid (gray), or slow MHC myofibers (black) were analyzed among populations of muscle fibers with non-expression of developmental MHC (A, B) or with expression of developmental MHC (C) in terms of fiber numbers (see Table 1) and fiber sizes (A-C). Note that larger sizes of slow MHC fibers were noticeable in populations of muscle fibers expressing fast and/or slow MHC(s) but not developmental MHC of affected muscles (B).

regenerating fibers, in contrast to the observation that fast MHC fibers consistently accounted for the majority of fibers in affected TC muscles, the affected diaphragms were mainly composed of hybrid and slow MHC fibers and the proportion increased gradually with age (Fig. 6C). These observations indicated that MHC expression in regenerating fibers was also different between affected TC muscle and diaphragm after 4 months old, although it was relatively similar in the two at 2 months old.

Temporal changes of MHC isoforms

To examine how progressive degeneration alters the composition of fiber types in affected skeletal muscles, we

detected myosin isoforms in TC muscles and diaphragms of CXMD₁ at various ages by electrophoretic gel separation (Fig. 7). Four MHC isoforms (I, IIA, IIX, and embryonic), which migrated on electrophoresis as IIA-embryonic-IIX-I from slowest to fastest [11,12], were detected in canine skeletal muscles (Fig. 7A). In affected TC muscles, type I, IIA, and embryonic isoforms were consistently detected at similar levels, but the level of type IIX MHC was lower than those in normal TC muscles after 2 months old. In contrast, type IIA MHC level decreased gradually in affected diaphragms with growth, and type I accounted for the majority of MHC components in animals over 6 months old. In addition, the embryonic isoform

Table 1: The numbers of myofibers co-expressing fast type, slow type, and/or developmental MHCs in skeletal muscles of a normal (10 months old) or a CXMD₁ dog (11 months old).

	TC			Diaphragm		
	Normal	Affected		Normal	Affected	
Developmental	-	-	+	-	-	+
Fast	265 (73%)	85 (40%)	155 (70%)	222 (48%)	12 (1%)	20 (3.6%)
Fast & slow	0 (0%)	38 (18%)	67 (30%)	0 (0%)	160 (16%)	370 (66.8%)
Slow	96 (27%)	88 (42%)	1 (0%)	243 (52%)	847 (83%)	164 (29.6%)
Total	361 (100%)	211 (49%)	223 (51%)	465 (100%)	1019 (65%)	554 (35%)

The numbers of fibers analyzed were results from a normal or an affected dog. MHC expression between two groups (normal, dMHC (-) vs affected, dMHC (-); affected, dMHC (-) vs affected, dMHC (+)), or between muscles (TC muscle vs diaphragm) was analyzed by Yates's chi-square test. Significant differences ($p < 0.05$) were detected in all tests.

decreased in affected diaphragms after 6 months old. These results were consistent with those of immunohistochemical analyses (Figs. 4 and 5). These observations suggested that type IIX and IIA fast fibers may be preferentially affected in TC muscle and diaphragm of CXMD₁, respectively. Furthermore, these observations suggested that muscle regeneration may deteriorate from

relatively younger age in the affected diaphragm, unlike TC muscle.

Discussion

To investigate the alterations in fiber types in skeletal muscles of a canine DMD model, we examined MHC expression in the TC muscle and diaphragm of CXMD₁ at various ages. Our results indicated that the influences of dys-

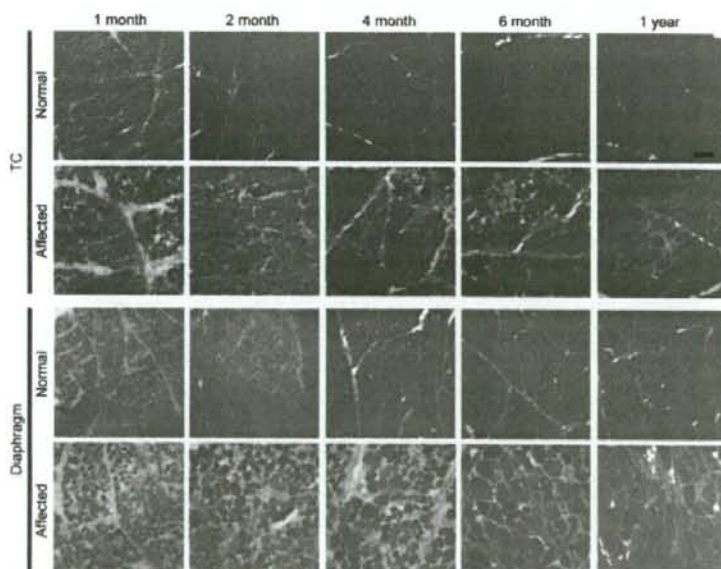


Figure 3
Representative histological findings in TC muscles and diaphragms of a normal or a CXMD₁ dog at 1, 2, 4, 6 months, and 1 year old. Note that severe degenerative lesions were observed from early ages in affected diaphragms, as compared with affected TC muscles. Bar: 200 μ m.

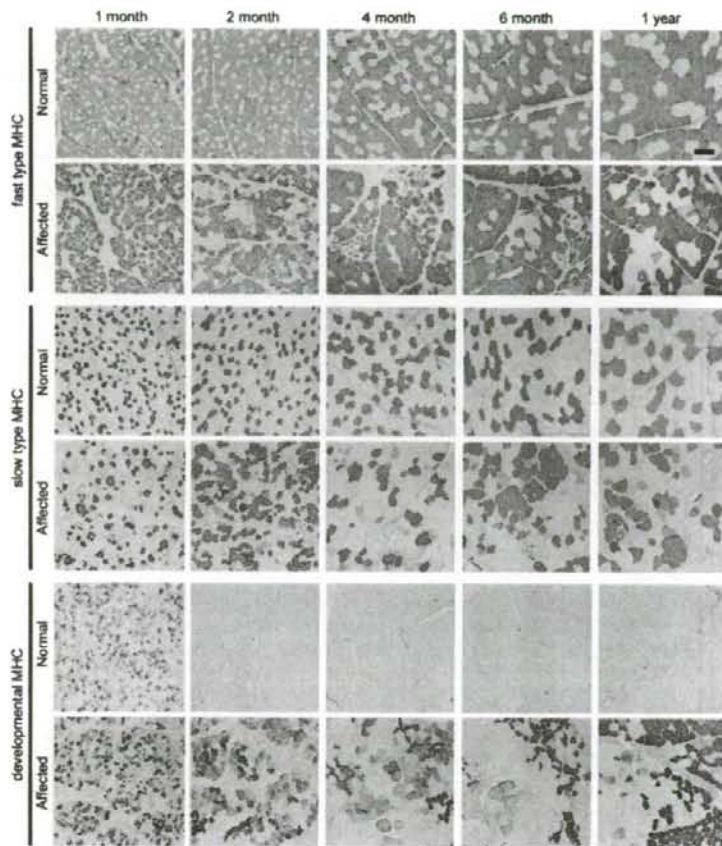


Figure 4
Expression of fast type, slow type, and developmental MHCs in TC muscles of a normal or a CXMD dog at 1, 2, 4, 6 months, and 1 year old. Note that there were no notable differences between expression levels of fast and slow type MHCs in normal and affected TC muscles. Bar: 200 μ m.

trophin deficiency on fiber type composition were significantly different between TC muscle and diaphragm.

To analyze MHC expression in details, we compared fiber type composition and fiber size distribution of MHC-expressing fibers between a normal dog (10 months old) and an affected dog (11 months old). In normal and affected dogs, body weight rapidly increased to approximately 9 kg at 4 months old, and then slightly increased to approximately 14 and 11 kg at 12 months old, respectively [5]. As body weight reflects muscle weight, muscle mass and fiber size would not extremely change in 1 month after 4 months old, especially in normal dogs. In fact, in TC muscles or diaphragms of normal dogs, there

were no significant differences among compositions of fiber types and MHC isoforms after 4 months old (Fig 6 and 7). In addition, we examined normal dogs at 11, 12 and 14 months old, and affected dogs at 10, 12, 13 and 15 months old. Normal muscles of adult dogs showed similar expression of fast type, slow type, or developmental MHC at all adult ages, and affected muscles also showed similar MHC expression at examined ages (data not shown). These observations implied that there would be no significant difference in MHC expression between at 10 and 11 months old, in both of normal and affected dogs.

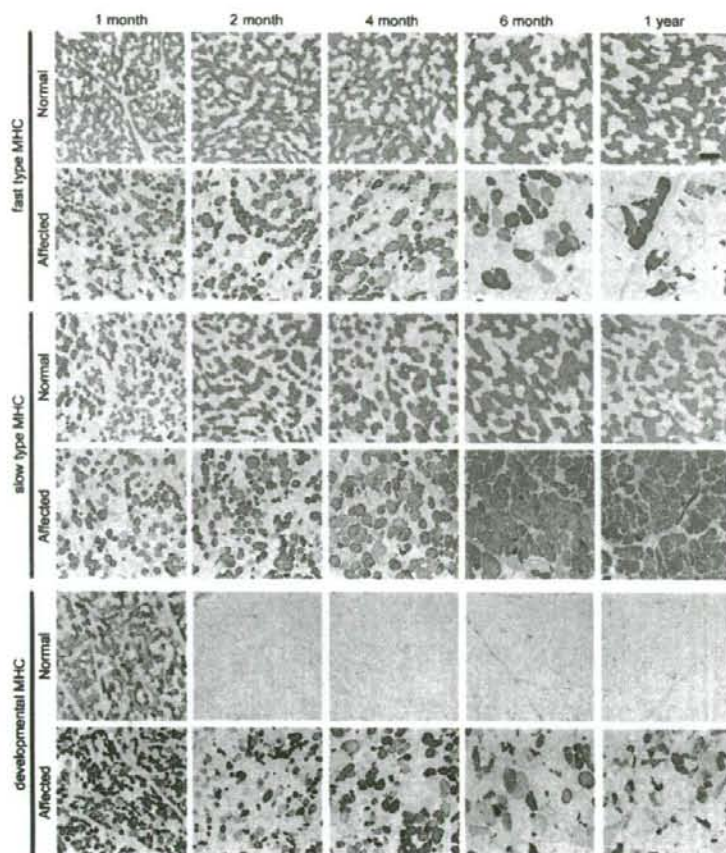


Figure 5

Expression of fast type, slow type, and developmental MHCs in diaphragms of a normal or a CXMD₁ dog at 1, 2, 4, 6 months, and 1 year old. Note that slow MHC fibers were increased markedly in the affected diaphragms after 6 months old, while fast MHC fibers were decreased. Bar: 200 μm.

Common features between TC muscle and diaphragm of CXMD₁

TC muscle and diaphragm of CXMD₁ shared the features that slow MHC fibers increased and enlarged selectively in non-regenerating populations, while fast type IIX or IIA MHC isoform decreased. Similar observations have been reported in skeletal muscles of the *mdx* mouse [13], GRMD [14], and human DMD [12,21]. In general, increasing and enlarging of slow fibers may be a consequence of adaptive responses by metabolic enzyme systems and energy consumption, because slow fibers have lower capacity for power output and consume less energy than fast fibers [22]. Our results also supported the

hypothesis that slow fibers would be more adaptable to dystrophic stress than fast fibers, to compensate for the reduced abilities of muscle function.

Two mechanisms were considered to explain the selective increase in slow fibers during progressive muscle degeneration. One possibility is that slow fibers may be more resistant to dystrophic stress than fast fibers, leading to selective survival of slow fibers. This was supported by the observation that slower muscle fibers contained significantly more utrophin, a homolog of dystrophin, in comparison to faster counterparts [23,24]. Another is transition of MHC isoforms, where type IIA or IIX MHC

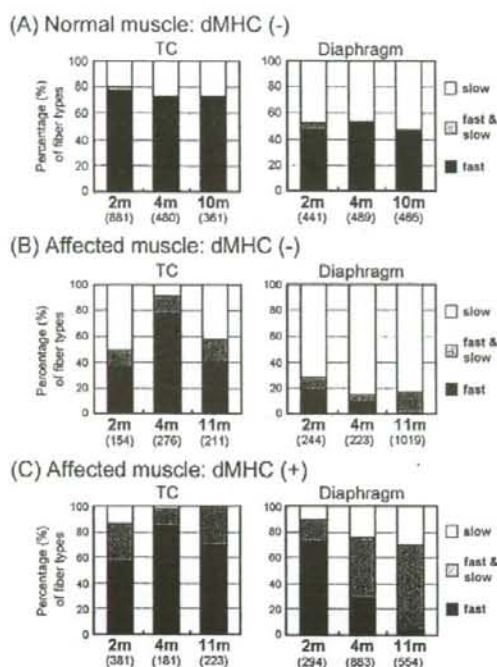


Figure 6

Proportions of fiber types in skeletal muscles of a normal or a CXMD₁ dog at various ages.

The numbers of fast (black), hybrid (gray), and slow MHC myofibers (white) among populations of myofibers without developmental MHC (A, B) and with developmental MHC (C) were counted in TC muscle and diaphragm of a normal (A) or an affected dog (B, C) at adolescent (2 or 4 months old) or adult stages (10 or 11 months old). The numbers under the ages show total fibers examined. MHC expression between two groups (normal, dMHC (-) vs affected, dMHC (-); affected, dMHC (-) vs affected, dMHC (+)), between muscles (TC muscle vs diaphragm), or among ages (2, 4, and 10 or 11 months) was analyzed by Yates's chi-square test. Significant differences ($p < 0.05$) were detected in all tests, except for no significant differences between 4 and 10 months old in normal TC muscles or diaphragms. Note that slow MHC fibers were consistently larger than other fibers, in populations of muscle fibers without developmental MHC of affected diaphragms. In populations of muscle fibers co-expressing developmental MHC and other MHC isoform(s), slow MHC and hybrid fibers were increased markedly in the affected diaphragm at 4 and 11 months old, unlike TC muscles.

isoforms could be transitioned to type I, as seen in hypertrophy and exercise [25]. MHC I, IIa, IIx, and IIb gene expression are known to be regulated by the calcineurin pathway [26,27]. Dystrophin deficiency may accelerate MHC tran-

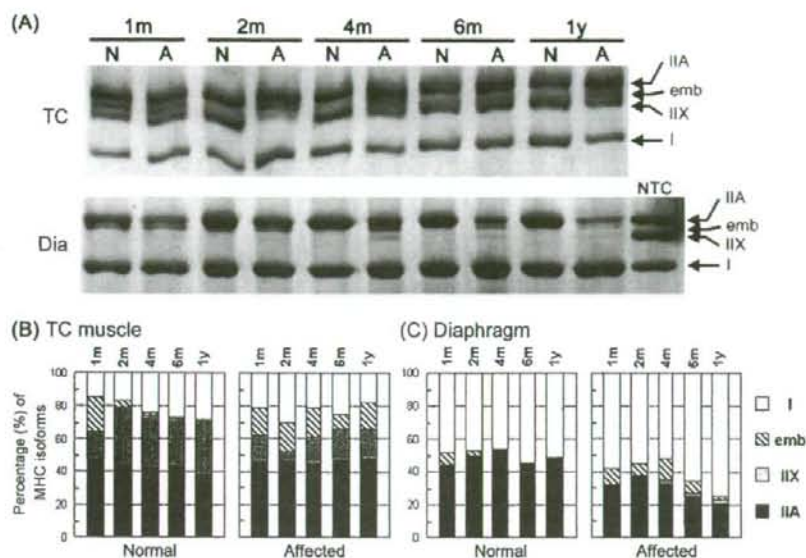
sition to slower types via calcineurin/NFAT signaling in skeletal muscles of CXMD₁, because calcineurin and activated NFATc1 protein content were higher in muscles from *mdx* than wild-type mice [28]. However, it remains possible that both mechanisms may be active at the same time, because the calcineurin/NFAT cascade can regulate not only the MHC promoters but also the utrophin A promoter [24,29,30].

Differences between TC muscle and diaphragm of CXMD₁

The CXMD₁ diaphragm developed severe degenerative lesions from earlier stages than TC muscle, which corresponded to previous reports [3,5,31]. In addition, dystrophic changes in the CXMD₁ diaphragm not only markedly altered the expression of fast and slow type MHCs but also decreased the amount of the developmental (embryonic and/or neonatal) MHC with growth, unlike affected TC muscle. Especially, fast MHC fibers disappeared and slow MHC fibers enlarged in the adult CXMD₁ diaphragm. The greater cross-sectional area of slow fibers in affected diaphragms might be due to hypertrophy in compensation for loss of fast fibers, relating to plasticity of muscle fibers, as mentioned above. The diaphragm keeps continuous contraction of muscle fibers without resting, while limb skeletal muscle regularly rests its movement. Therefore, replacement with slow fibers may be particularly enhanced in the diaphragm rather than TC muscle, depending on pathological severity and contractile activity of skeletal muscles.

Fiber type determination and fiber type-specific gene expression are regulated by multiple signaling pathways and transcription factors. As partially described above, a key mediator, calcineurin, plays an important role in acquisition of fiber phenotype [29,30] and may induce not only transition of MHC isoforms from faster to slower types but also transformation of myofiber phenotypes in mouse or rat muscles [26,27,32]. In addition, calcineurin signaling activity was greater in the diaphragm than in the tibialis anterior muscle of the *mdx* mouse [28]. Therefore, replacement with slow fibers may be up-regulated to a greater extent in the diaphragm than in the TC muscle of CXMD₁.

We also showed age-related changes of MHC expression in affected diaphragms after 6 months old, in contrast to TC muscles (Fig 4, 5 and 7). In addition, fiber type compositions in non-regenerating or regenerating fibers were also different between the TC muscle and the diaphragm, depending on age. In non-regenerating fibers of affected TC muscles, fast MHC fibers at 4 months old was higher than those at 2 and 11 months old (Fig. 6B). It might be partially involved in pathological changes that degenerative lesions appeared obviously in affected TC muscles after 4 months old, as described previously [3,5,31]. In

**Figure 7**

MHC isoforms in skeletal muscles of normal and CXMD₁ dogs. (A) Electrophoretic separation of MHC isoforms in TC muscle and diaphragm. Myosin was extracted from muscles at various ages (1, 2, 4, 6 months, and 1 year old), and aliquots of 0.4 μ g of protein were separated on 8% SDS-polyacrylamide gels containing 30% glycerol. Four MHC isoforms (I, IIX, IIA, and embryonic) were detected. NTC: normal TC muscle at 1 year old. Note that MHC type I increased in the affected diaphragm after 6 months old. (B) Quantitative analysis of MHC isoforms. MHC expression between two groups (normal vs affected) or among ages (1, 2, 4, 6 months and 1 year) was analyzed by Yates's chi-square test. Significant differences ($p < 0.05$) were detected between normal and affected groups in TC muscles after 2 months old or in diaphragms after 4 months old, and between 1 and 2 months old in normal TC muscles.

regenerating fibers of the CXMD₁ diaphragm, the proportion of myofibers expressing slow type MHC increased markedly after 4 months old (Fig. 6C). These results suggested that MHC expression in TC muscle and the diaphragm of CXMD₁ would be influenced by different mechanisms after 4 months old. These age-dependent MHC expression might be related to body growth, particularly increasing of muscle mass. One possibility is participation of insulin-like growth factor (IGF)-1, which is important for postnatal growth of skeletal muscles [33] and can activate multiple Ca²⁺-dependent signaling pathways, including the calcineurin/NFAT pathway [30]. When growth rate of body weight decreases after 4 months old [5], signaling activity of IGF-1 might reduce and MHC expression might be regulated predominantly by alternative signaling pathways.

Comparison among *mdx*, CXMD₁, and DMD diaphragms

MHC expression in normal skeletal muscle has been well studied in mice [15,34], dogs [11], and humans [35]. In normal dogs, the proportions of fiber types in TC muscle

were relatively similar to those in the representative tibialis anterior muscles of mice and humans. In the diaphragm, however, the proportion of fiber types differed markedly among these species. The murine diaphragm is composed mainly of fast type IIA and IIX isoforms [15,34], but the canine diaphragm consists of equal populations of slow type MHC I and fast type MHC IIA [11], as also shown in our study. In normal human diaphragm, the distribution of myosin isoforms has been estimated that types I, IIA, and IIX account for approximately 45%, 40%, and 15%, respectively [35]. Thus, the proportions of MHC isoforms in the diaphragm of healthy dogs are much closer to those of humans than those of mice.

Some groups have studied expression profiles of MHC isoforms in the diaphragm of the *mdx* mouse. The *mdx* diaphragm shows increases in MHC type I fibers and elimination of type IIX population at 2 years old, but not at young ages (3 to 6 months old) [13,15,34]. In contrast to the *mdx* diaphragm, that in CXMD₁ exhibited drastic changes even in younger animals (6 months old). On the

other hand, there is no direct information available regarding the changes in fiber type composition in the diaphragm in human DMD. In addition, there is an important difference of MHC expression even in limb skeletal muscles between large mammals (including dogs and humans) and mammals with smaller body mass, especially rodents. The former do not express the fastest MHC IIB isoform in limb muscles [10,11,36], while it is abundantly expressed in the latter [34]. Therefore, changes/adaptations in skeletal muscles of dogs with muscular dystrophy are likely to be more relevant to human DMD, than that in the *mdx* mouse. As it is difficult to examine the diaphragms of DMD patients, it would be important to investigate the differences between murine and canine models for understanding the mechanisms of respiratory failure in human DMD.

Conclusion

Based on fiber type classification using MHC expression, we demonstrated the predominant replacement with slow fibers and reduced muscle regeneration with progression of muscular dystrophy in the diaphragm of a canine DMD model, but these phenomena were much less strict in affected TC muscle. In addition, the expression profiles of MHC isoforms in the CXMD₁ diaphragm were evidently different from those of the *mdx* mouse. Our results indicated that dystrophic dog is a more appropriate model than a murine one for human DMD, and would be useful for investigation of the mechanisms of respiratory failure in DMD, as well as pathological and molecular biological backgrounds, and therapeutic effects in clinical trials.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KY designed the study, carried out the pathological and immunohistological examinations, and drafted the manuscript. AN participated in interpretation of data, and helped to draft the manuscript. TH participated in coordination of the study. ST participated in the design, planning, and coordination of the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Levodopa in the early treatment of Parkinson's disease

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Abstract

L-dopa has many advantages as initial therapy for Parkinson's disease (PD). It is safer, more efficacious, associated with fewer adverse effects, few interactions, easier for patients to use and for clinicians to prescribe, and cheaper than dopamine (DA) agonists. Although L-dopa is more likely than DA agonists to introduce motor fluctuations and dyskinesia, L-dopa is also more effective in improving motor function. Furthermore, there is no long-term benefit from delaying L-dopa based on the risk of motor complications or psychiatric symptoms. Many investigations have shown that L-dopa does not accelerate disease progression. Now is the time to re-evaluate L-dopa for initial treatment of PD. © 2008 Elsevier Ltd. All rights reserved.

Keywords: Parkinson's disease; L-dopa; DA agonist; Motor fluctuation, Dyskinesia

1. Introduction

Although it is recommended that dopamine (DA) agonists should be chosen as initial treatment for Parkinson's disease (PD), it is time to re-evaluate the use of levodopa for initial treatment of PD.

While many dopaminergic drugs have been introduced, L-dopa therapy has remained the gold standard for symptomatic treatment of PD. L-dopa is safer, more efficacious, associated with fewer adverse effects, has few interactions, is easier for patients to use and for clinicians to prescribe, and it is cheaper than DA agonists. Despite these advantages, many previous guidelines (Fig. 1) [1,2] have stated that for early stage patients with PD it is appropriate to start treatment with a DA agonist unless the patient is either older than 75 years or has dementia. The rationale for these recommendations has been that (1) L-dopa may accelerate disease progression, and (2) DA agonists are less likely to induce motor fluctuation.

2. Is L-dopa really neurotoxic?

Concern that exogenous L-dopa may be neurotoxic and contribute to the progression of PD arises from the oxidative stress hypothesis of PD [3]. Many *in vitro* studies have demonstrated that the addition of L-dopa or DA to cultured dopaminergic neurons increased cell death [4,5]. However, these experiments were performed under non-physiologic conditions; the concentrations of L-dopa and DA in these experiments were high ($>5 \mu\text{M}$) and exceeded what would be expected in the brains of patients treated with therapeutic doses ($<2 \mu\text{M}$). Furthermore, some *in vitro* studies have shown that L-dopa and DA are neuroprotective when neurons are co-cultured with glial cells [6,7].

The ELLDOPA study, conducted to show whether L-dopa is toxic for PD patients, was a large, double-blind, randomized, controlled clinical trial comparing three different doses (150, 300, 600 mg per day) of L-dopa with placebo in patients with early PD [8]. At the end of the trial (after a 2-week washout period), the mean Unified Parkinson's Disease Rating Scale (UPDRS) score of patients treated with L-dopa was better than that of the placebo group. The mean UPDRS score of the highest dosage group was the best, even after the washout period. These results suggest that L-dopa is not toxic, and may even be neuroprotective. This study also included evaluation with $\beta\text{-CIT}$ SPECT

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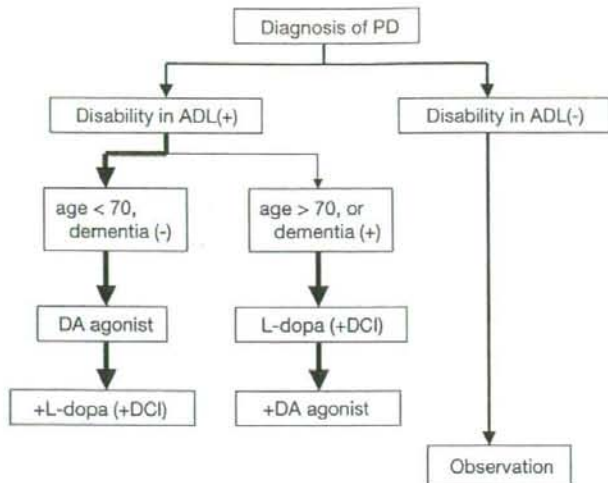


Fig. 1. Guideline for Parkinson's disease (early stage) made by Japanese Society for Neurology, 2002. ADL, activities of daily living; DCI, decarboxylase inhibitor.

imaging, as a marker for intact nigrostriatal dopaminergic neuronal functioning. The imaging studies showed that there was a larger decrease in β -CIT uptake in patients treated with L-dopa in a dose-response manner. These clinical and imaging results suggested that β -CIT SPECT imaging is not appropriate for evaluating the neuroprotective ability of the drugs. Up to that time, several studies had shown that DA agonists are more protective than L-dopa using β -CIT SPECT [9,10] or F-DOPA PET [11]. The results of the ELLDOPA study permitted re-evaluation of the results of these studies, and there is now a consensus that L-dopa does not accelerate disease progression [12].

3. L-dopa is more likely than DA agonists to induce motor fluctuations and dyskinesia

Several clinical studies [9,11] have shown that treatment with DA agonists is less likely to induce motor complications than treatment with L-dopa. These studies also showed that L-dopa monotherapy improves activities of daily living and motor function to a greater degree than DA agonists (plus later optional L-dopa). What is of most importance to our patients? The severity of both motor fluctuation and dyskinesia in these studies was low. For example, in the CALM-PD study (4 years) the percentage of disabling dyskinesia was 4.4% in the pramipexole group and 6.9% in the L-dopa group [13]. Furthermore, retrospective investigations failed to show any long-term benefit from delaying L-dopa based on the risk of motor complications, dementia, or psychiatric symptoms [13–16].

Apart from motor complications, the frequency of other common side effects is less with L-dopa than with DA agonists. For example, hallucinations are about three times more likely to occur with ropinirole or pramipexole than with L-dopa [17]. Somnolence, edema, and cardiac valvular fibrosis (pergolide, cabergoline) are also more frequent with

DA agonist treatment than with L-dopa [17]. In Japan, those who are taking ropinirole or pramipexole are prohibited from driving because of the risk of sudden onset sleep. Can the incidence of motor fluctuation be reduced only at the expense of improvements in motor function and activities of daily living, and of other side effects such as hallucination, sudden onset sleep, and fibrosis?

4. Mechanism of wearing-off

Disease progression is associated with "wearing off" of therapeutic benefit and the appearance of unpredictable treatment responses, resulting in complex "on-off" response fluctuations. These arise in between doses of L-dopa because the patient no longer has the ability to store dopamine. The other factors driving development of response fluctuations are changes in peripheral L-dopa pharmacokinetics and in post-synaptic function that accompany large-dose and long-term L-dopa therapy [18]. Jenner and colleagues reported on the relation between the amount of lesion and the development of dyskinesia and motor fluctuation by using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) marmoset model [19]. In primates with 50% lesions (model of early PD), L-dopa produced an antiparkinsonian response with no induction of dyskinesia. In the group with 75% lesions, L-dopa produced an antiparkinsonian response with a gradual development of dyskinesia. In the >90% lesion group, L-dopa produced a pulsatile antiparkinsonian response and rapid induction of severe dyskinesia.

Pathological examination in patients with PD demonstrates an exponential loss of nigral pigmented neurons [20]. At 5 years from symptom onset, about 50% of the pigmented neurons remain, compared with age-adjusted controls, and at 10 years 30% remain. The presymptomatic phase of PD, dating from the onset of neuronal loss, was estimated to be about 5 years. Therefore, at the initial symptomatic stage

of the disease there is little possibility of developing motor fluctuation and dyskinesia as long as the appropriate dose of L-dopa is used. In fact, the ELLDOPA study showed the incidence of motor complications in the L-dopa 150 mg and 300 mg groups to be almost equal to that in the placebo group [8].

5. The advantage of L-dopa as initial therapy for Parkinson's disease

The initial use of L-dopa for the symptomatic treatment of PD has many advantages over DA agonists: (1) L-dopa has significantly greater efficacy than DA agonists in alleviating the motor symptoms of PD and improving activities of daily living. (2) Titration of L-dopa to therapeutic levels is much easier and faster than that of DA agonists. (3) L-dopa is much less likely to induce hallucinations, somnolence, edema, or constipation compared to DA agonists. (4) There is a clear cost benefit to using L-dopa. (In Japan, L-dopa + decarboxylase inhibitor 300 mg/day costs about 1 US dollar per day; pramipexole 4.5 mg/day about 16 US dollars; and ropinirole 15 mg/day 26 US dollars.) However, late-developing motor fluctuation and dyskinesia deserve consideration, and the potential for other common side effects such as hallucinations and sleep attacks must also be factored into the treatment decision. Furthermore, early in the course of the disease, L-dopa provides an enduring response that can last several days [21].

There is much evidence to show that DA agonists are efficacious in controlling L-dopa motor fluctuations (as later adjunctive therapy). However, another option is to initiate treatment with levodopa, adding a DA agonist after the first sign of developing motor complications has appeared (early combination).

6. Continuous stimulation by using L-dopa

Continuous daytime intrainestinal infusion of L-dopa can diminish motor complications [22]. This shows that motor complications can be improved by changing the pharmacokinetics of L-dopa. For example, catechol-*O*-methyltransferase

(COMT) inhibitors can extend the half-life of serum L-dopa concentration. The duration of L-dopa efficacy can also be extended by taking L-dopa after a meal (Fig. 2). Long-term L-dopa therapy increases the peak L-dopa concentration (C_{max}) and decreases its half-life ($T_{1/2}$) [23]. C_{max} is decreased and $T_{1/2}$ is increased by taking L-dopa after a meal compared to taking it before meals. Therefore, taking L-dopa after meals not only extends effective time but also decreases dyskinesia. By taking L-dopa after meals, the dose of L-dopa can be increased, but the risk of dyskinesia can be decreased. If needed, a low dose of L-dopa may be taken before meals for immediate improvements, with the remainder of the dose taken after meals.

There is much evidence for the efficacy of DA agonists as adjunctive therapy in controlling L-dopa motor fluctuations and dyskinesia [24]. Monoamine oxidase (MAO) B inhibitors [24] and zonisamide [25] may also be used as adjunctive therapy to improve motor fluctuations.

7. Initial symptomatic treatment for early Parkinson's disease

It is appropriate to start treatment of PD with either L-dopa or DA agonists. As highlighted by the American Academy of Neurology practice parameter [17], the choice of initial treatment depends on the relative importance for the patient of improving motor disability and limiting adverse events versus the possibility of lowering the risk of developing long-term motor complications.

The frequency of the development of wearing-off depends on the age of disease onset. Younger-onset patients (younger than 50 years at onset) are more prone to severe dyskinesia and motor fluctuation, while patients older than 70 years at symptom-onset rarely develop disabling dyskinesia and motor fluctuation. Older patients may be more prone to develop hallucinations and other common adverse effects. Therefore, L-dopa is preferred for elderly patients as initial treatment. In younger patients, DA agonists are preferred, but if the patient is at risk of losing his or her job owing to motor disability, L-dopa should be started. Patients whose age of onset is between 50 and 70 years can be prescribed

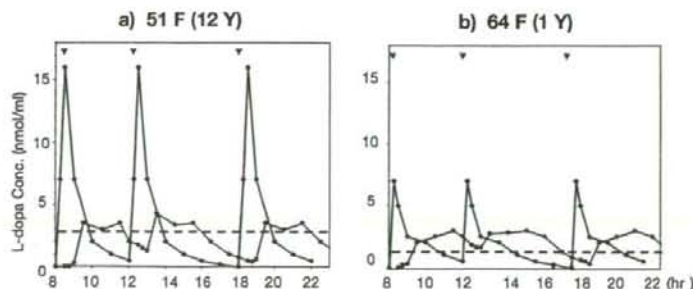


Fig. 2. Effects of a meal on L-dopa pharmacokinetics. (a) 51-year-old woman with 12 years' duration of Parkinson's disease. (b) 64-year-old woman with 1 year duration of Parkinson's disease. A tablet of L-dopa 100 mg + benserazide 20 mg was taken. Black curve: just before meal, red curve: after meal 3 times a day. ∇ : meals (at 08.00, 12.00, 18.00 hours). Dashed line: the concentration of effective threshold estimated by clinical symptoms. C_{max} is 2–3 times higher when L-dopa was taken before meals than after meals. L-dopa pharmacokinetics are more stable when taken after meals than before meals.

either L-dopa or a DA agonist. Treatment selection should reflect the patient's needs and under-treatment should be avoided.

Conflict of interest

The author has no conflict of interest to report. No funding applicable.

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LRRK2 P755L variant in sporadic Parkinson's disease

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Abstract Parkinson's disease (PD) is a neurodegenerative disorder of unknown etiology with probable involvement of genetic-environmental factors. The majority of PD cases (approximately 90–95%) are sporadic, while familial cases account for approximately 5–10% of PD. In a recent report, a heterozygous *LRRK2* P755L mutation within *LRRK2* exon 19 was found in 2% of Chinese sporadic PD patients and in 0% of normal controls or Caucasians, suggesting that the mutation is disease-associated with ethnic specificity. To further evaluate the role of *LRRK2* P755L variant in sporadic PD, we performed direct sequencing of *LRRK2* exon 19 in

501 Japanese sporadic PD patients (male 249, female 252, aged 28–92 years, mean 65.0 years) and 583 controls of the Japanese general population as an extended association study. In this group, we found six patients (6/501 = 1.2%) and eight controls of the general population (8/583 = 1.6%) with a heterozygous P755L variant ($P = 0.80$, $\chi^2 = 0.064$). No other variants were found in exon 19. Together with previous reports, our extended case-controlled study of large sample size suggests that *LRRK2* P755L is a non-disease-associated polymorphism in PD patients.

Keywords Parkinson's disease · Genetics · *PARK8* · *Leucine-rich repeat kinase 2 (LRRK2)* · Polymorphism · Association study · Japanese · Ethnic background

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Introduction

Parkinson's disease (PD, OMIM #168600) is the second most common neurodegenerative disorder next to Alzheimer's disease. The clinical features are characterized by levodopa-responsive parkinsonism, such as rigidity, resting tremor, bradykinesia, and postural instability. Although the cause of PD remains unclear, genetic-environmental interaction is suggested for the development of the disease. One of the autosomal-dominant forms of PD, *PARK8*, was originally mapped from a Japanese Sagamihara family (Funayama et al. 2002) and *LRRK2 (PARK8; leucine-rich repeat kinase 2, OMIM #609007)* was identified as the causative gene for *PARK8*-linked PD (Paisán-Ruiz et al. 2004; Zimprich et al. 2004). Among *LRRK2* mutations, the most common *LRRK2* G2019S mutation in North Africans and Ashkenazi Jews has shown ethnic differences among Caucasian, Japanese, and Chinese (Nichols et al. 2005; Gilks et al. 2005; Lesage et al. 2006; Tomiyama et al.

2006; Tan et al. 2005). On the other hand, *LRRK2* G2385R variant has recently been found the most common genetic risk factor among Chinese and Japanese, but not Caucasians (Di Fonzo et al. 2006; Funayama et al. 2007; Tan et al. 2007; Farrer et al. 2007). Moreover, in a recent report (Wu et al. 2006), a heterozygous *LRRK2* p.P755L (c.2264c > t, rs34410987) mutation within *LRRK2* exon 19, corresponding to a predicted ankyrin-repeat-like domain of *LRRK2*, was found in 2% (12/598) of Chinese sporadic PD and 0% (0/765) of Chinese normal controls, suggesting its association with the disease. However, *LRRK2* P755L was reported as a polymorphism (3% of 92 normal controls) in the dbSNP database of Taiwanese. Thus, to determine the frequency and the role of *LRRK2* P755L in Asian PD, we screened for *LRRK2* exon 19 in Japanese sporadic PD patients.

Subjects and methods

The nucleotide sequences of *LRRK2* exon 19 were determined by direct sequencing in 501 sporadic Japanese PD patients and 583 controls of the Japanese general population (Table 1). All blood samples and clinical information were obtained by the attending neurologists after obtaining informed consent from their patients. The study was approved by the ethics review committees of Juntendo and Osaka Universities. Diagnosis of PD was made by the attending neurologists based on the presence of parkinsonism and good response to anti-PD treatment. Controls of the Japanese general population were evaluated by neurologists to ensure none of them had PD. DNA was prepared using standard methods. They were amplified by polymerase chain reaction (PCR) of exon 19 and sequenced using BigDye Terminator Chemistry and ABI310 and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences of the primers, conditions of PCR, and conditions of sequencing were based on a previous report (Zimprich et al. 2004).

Results

We found 6 patients (6/501 = 1.2%) and 8 controls of the Japanese general population (8/583 = 1.6%) with a heterozygous P755L variant ($P = 0.80$, odds ratio = 1.15, 95% CI: 0.40–3.32, $\chi^2 = 0.064$) in *LRRK2* exon 19 (Table 2). No other variants were found in exon 19.

Discussion

The purpose of the present study was to clarify the role of an ethnic-specific variant in the causative gene for PD. Although PD is considered a heterogeneous disease with genetic-environmental interaction, some cases certainly exhibit a Mendelian-inherited disease or are associated with strong genetic and ethnic background. Indeed, the reported frequency of *LRRK2* G2385R was higher in Asian sporadic PD patients than in controls (Di Fonzo et al. 2006; Funayama et al. 2007; Tan et al. 2007), although this is not the case in Caucasians. Moreover, Wu et al. (2006) in Nanjing, China, recently reported that a heterozygous *LRRK2* P755L mutation was found in 2% (12/598) of Chinese sporadic PD and 0% (0/765) of normal controls, whereas none (0/463) of the Caucasian PD patients had this mutation (Deng et al. 2007), suggesting ethnic differences, like *LRRK2* G2385R. However, our results of large case-controlled study in Japanese revealed that *LRRK2* P755L is a non-disease associated polymorphism. Consistent with our data, this variant was present at similar frequency in Taiwanese PD patients (7/578 = 0.99%) and Taiwanese normal controls (10/339 = 0.97%) (Di Fonzo et al. 2006). Furthermore, the latest report in the Chinese population in Singapore showed the absence of segregation and association of P755L with PD (case 4/204 = 2.0%, control 6/235 = 2.6%, $P = 0.76$) (Tan et al. 2008). These findings might be based on ethnic or native differences in human migration history or human genetics.

We reported previously that the most common *LRRK2* G2019S mutation in Mendelian-inherited and sporadic PD

Table 1 Profile of analyzed samples in this study

Parameter	Patients	Controls of general population
Total sample, <i>n</i> (%)	501 (100)	583 (100)
Male, <i>n</i> (%)	249 (49.7)	312 (53.5)
Female, <i>n</i> (%)	252 (50.3)	271 (46.5)
Age at sampling (years) ^a	65.0 ± 9.6 (28–92)	45.0 ± 17.0 (21–98)
Male ^a	64.3 ± 10.2 (28–92)	43.6 ± 15.0 (22–92)
Female ^a	65.4 ± 9.9 (28–92)	46.8 ± 19.0 (21–98)
Age at onset (years) ^a	58.0 ± 10.5 (20–88)	
Male ^a	57.7 ± 10.9 (20–88)	
Female ^a	58.3 ± 10.1 (25–82)	

^a Data are mean ± SD (range)

Table 2 Allele frequency of *LRRK2* c. 2264C > T (p. P755L) in Japanese patients with Parkinson's disease and controls of general population

	Genotype, n (%)			Allele, n (%)		χ^2 ^a	OR (95% CI)
	C/C	C/T	T/T	C	T		
Patients (n = 501)	495 (98.8)	6 (1.2)	0 (0)	996 (99.4)	6 (0.6)	0.06	1.15 (0.40–3.32)
Controls of general population (n = 583)	575 (98.6)	8 (1.4)	0 (0)	1,158 (99.3)	8 (0.7)		

^a Compared with the control

OR odds ratio, CI confidence interval

was rare in Asians compared to North Africans or Caucasians (Tomiyama et al. 2006). *LRRK2* variants are reported to spread worldwide with some ethnic differences among each variant, such as R1441G, R1441C, R1441H (exon 31, ROC domain), G2019S, I2020T (exon 41, MAPKKK domain), and G2385R (exon 48, WD40 domain) (Mata et al. 2005). Since *LRRK2* consists of as many as 51 exons, it is important to decide which exon(s) of this gene should be screened first for efficient analysis of mutation in patients with various ethnic backgrounds. In this regard, *LRRK2* exon 41 and 31 are reasonable to be screened first; however, exon 19 is not likely a candidate exon for causative mutation screening in PD. In addition, although MAPKKK and ROC domain are reported to be associated with kinase activity of *LRRK2* (Paisán-Ruiz et al. 2004; Zimprich et al. 2004; Smith et al. 2006), the existence and the role of the predicted ankyrin repeat-like domain in *LRRK2* have not been established yet.

So far, *LRRK2* P755L as well as G2385R variants have been found in only Chinese, Taiwanese, and Japanese (Asians) with similar frequencies in some Asians, but have not been found in Caucasians. Thus, these variants could occur independently in very ancient Asians with a single founder effect (Farrer et al. 2007). Although the HapMap project has been very successful, the presence of ethnic differences among *LRRK2* variants such as G2019S, R1441G, G2385R, and P755L suggest that further establishment of ethnic-specific or native-specific data is essential for more accurate SNP analyses and genome-wide association studies.

Conclusion

Our extended association study in Japanese with large sample size suggests that *LRRK2* P755L is a non-disease-associated polymorphism in PD patients.

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