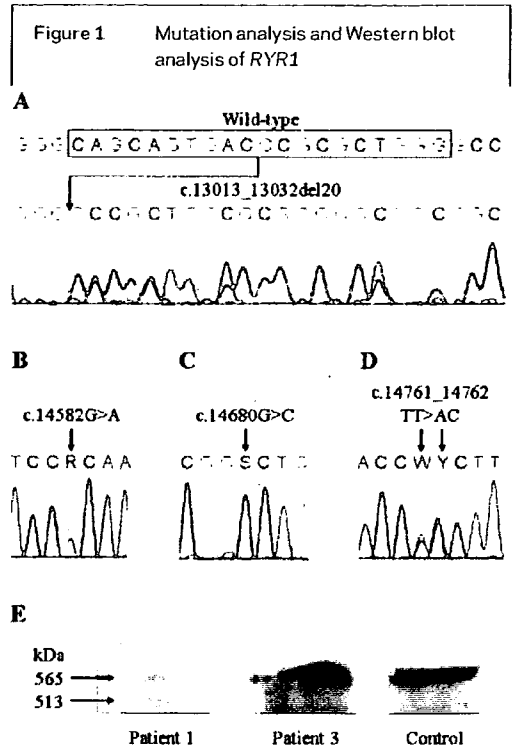



Figure 1 Mutation analysis and Western blot analysis of *RYR1*

(A) A novel deletion mutation of c.13013_13032del20 (p.A4338fs) is identified in exon 91 in Patient 1. Deleted nucleotides (enclosed in box) are shown in wild-type sequence. (B) In Patient 2, a known missense mutation c.14582G→A (p.R4861H) is detected in exon 101. (C) A novel missense mutation of c.14680G→C (p.A4894P) is identified in Patient 3 in exon 102. (D) In Patient 4, substitution of two consecutive nucleotides c.14761_14762TT→AC (p.F4921T) is identified in exon 102. Arrows mark the site of mutation. (E) Western blot analysis of muscles from Patient 1, Patient 3, and control. All show a 565-kd band corresponding to the predicted size of full-length *RYR1* (upper arrow). Only Patient 1 has a smaller sized band (about 513 kd) corresponding to the truncated *RYR1* mutant (lower arrow).

**RESULTS Mutation screening.** Four of 10 patients (40%) had heterozygous sequence variations predicted to change amino acids in the C-terminal domain of RYR1 (figure 1, A through D). In Patient 1, we identified a 20-bp deletion (c.13013_13032del-CAGCAGTGACGCGCGCTGGG, p.A4338fs) in exon 91, which resulted in a premature stop codon at the 4,575th amino acid. The presence of the deletion mutation was confirmed by sequencing of the cloned fragments. No family members including the parents and siblings carried the mutation, suggesting that the mutation in Patient 1 is a de novo mutation. Patient 2 had a missense mutation (c.14582G→A, p.R4861H) in exon 101, which had been previously reported in patients with CCD.[14] In Patient 3, a novel missense mutation of c.14680G→C (p.A4894P) was identified in exon 102. Patient 4 had a substitution of two consecutive nucleotides (c.14761_14762TT→AC, p.F4921T) in exon 102, which was previously reported in his father with CCD.[6,11] We confirmed the two-nucleotide change in one allele and the absence in the other by sequencing of the cloned fragments in Patient 4. All three amino acids replaced by missense mutations, R4861, A4894, and F4921, were highly conserved across the *RYR1* species including human, pig, rabbit, mouse, frog, and *C. elegans* (data not shown). These substitutions were not found in either 300 Japanese control chromosomes or in the Japanese Single Nucleotide Polymorphisms database.[15]

The substitution c.11266C→G (p.Q3756E) in exon 79 previously reported as nonpathogenic[17] was found in 4 of 10 patients: 1 with *RYR1* mutation and 3 without *RYR1* mutation. This substitution was also reported in the Japanese population diversity to be 11.4% in the Japanese Single Nucleotide Polymorphisms database . Fifteen silent single-nucleotide polymorphisms were also identified (data not shown). For the six patients without *RYR1* mutation, we were able to amplify and sequence the C-terminal domain of RYR1 in muscle cDNA and confirmed the absence of any mutation including aberrant splicing.

No mutations were found in either *FKBP1A* or *CACNA1S*. Two patients with congenital myopathy with marked type 1 fiber predominance did not have any mutation in *RYR1*.

We also tried to sequence the C-terminal domain of RYR1 using DNA from the original three patients first reported to have CNMDU1.[1] However, as only paraffin-embedded muscles were available, the quality of DNA did not allow us to successfully amplify the regions except for exons 96 and 100 wherein no mutation was found.

**Western blot analysis.** To know whether the truncated protein is expressed in Patient 1, we performed Western blot analysis. As expected, the muscle from Patient 1 showed two bands: a 565-kd band of predicted size of wild-type RYR1 protein and a 513-kd band, which is the predicted size of mutant *RYR1* (figure 1E, left lane). The lower bands were not observed in samples from the other patient and control (figure 1E, center and right lanes).

**Clinical features.** None of the four patients with C-terminal mutations in *RYR1* showed mental retardation (table). Moreover, no severe clinical incident during the perinatal stage was observed in this group. As described above, two patients had family history of neuromuscular disease and the father of Patient 4 was reported to have CCD.[6]

Table Clinicopathologic features of patients with and without *RYR1* mutations

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Nucleotide changes | c.13013_13032del20 | c.14582G→A | c.14680G→C | c.14761_14762TT→AC | – | – | – | – | – | – |
| Protein mutations | p.A4338fs | p.R4861H | p.A4894P | p.F4921T | | | | | | |
| Exons | 91 | 101 | 102 | 102 | | | | | | |
| Age at biopsy/sex | 3 y 5 mo/M | 6 mo/M | 6 y/F | 2 y 9 mo/M | 5 mo/M | 7 mo/M | 8 mo/M | 11 mo/F | 3 y 10 mo/M | 13 y/F |
| Age at last clinical examination | 8 y | 6 mo | 6 y | 11 y | 2 y 9 mo | 5 y 3 mo | 3 y 5 mo | 5 y 5 mo | 3 y 10 mo | 21 y |
| Family history | – | + | NA | + | NA | – | – | – | – | – |
| Poor fetal movement | – | – | + | – | NA | + | + | + | + | – |
| Asphyxia | – | – | – | – | + | + | + | + | – | – |
| Infantile hypotonia | NA | – | + | – | + | + | + | + | + | + |
| Poor sucking | + | + | + | – | + | NA | + | + | + | + |
| Respiratory distress | + | – | – | – | + | + | + | + | – | + |
| Muscle weakness | G | + | P | P | G | G | G | P | P | P |
| Delayed motor milestones | + | – | + | + | + | + | + | + | + | + |
| Mental retardation | – | – | – | – | + | + | + | + | + | – |
| Facial muscle involvement | + | – | + | – | NA | + | + | + | + | + |
| High arched palate | + | – | NA | – | + | + | + | + | + | + |
| Skeletal deformity | FC | JC | Lo, HD | – | FC | FC, Sc, HD | Sc, JC | Sc, JC | JC | Sc |
| Biopsied muscle | BB | QF | AL | BB | NA | BB | BB | NA | QF | BB |
| Total no. of muscle fibers | 1,770 | 1,887 | 1,709 | 1,609 | 1,665 | 2,056 | 1,546 | 1,414 | 1,558 | 1,252 |
| Type 1 fibers, n (%) | 1,757 (99.3) | 1,886 (99.9) | 1,709 (100) | 1,609 (100) | 1,663 (99.9) | 2,949 (99.7) | 1,537 (99.4) | 1,408 (99.6) | 1,557 (99.9) | 1,249 (99.8) |
| Type 2 fibers, n, 2A/2B/2C | 0/11/2 | 0/0/1 | 0/0/0 | 0/0/0 | 0/0/2 | 0/0/7 | 0/1/8 | 0/0/6 | 0/0/1 | 0/2/1 |
| Internal nuclei, % | 0.4 | 0.2 | 0.2 | 0.2 | 1.7 | 0.2 | 0.4 | 4.0 | 0.2 | 0.3 |
| Endomysial fibrosis | Mild | Minimal | Mild | Mild | Mild | Mild | Marked | Moderate | Minimal | Minimal |

G = generalized; P = proximal; FC = funnel chest; JC = joint contracture; HD = hip dislocation; Lo = lordosis; Sc = scoliosis; (+) = present; (–) = absent; BB = biceps brachii; QF = quadriceps femoralis; AL = adductor longus; NA = no information was available

In contrast, five of six patients without mutations in *RYR1* had mental retardation except Patient 10. Severe respiratory distress, with asphyxia or infection necessitating mechanical support, was observed in five patients. None had family history of any neuromuscular disease. Myopathic facies and high arched palate were predominant in this group.

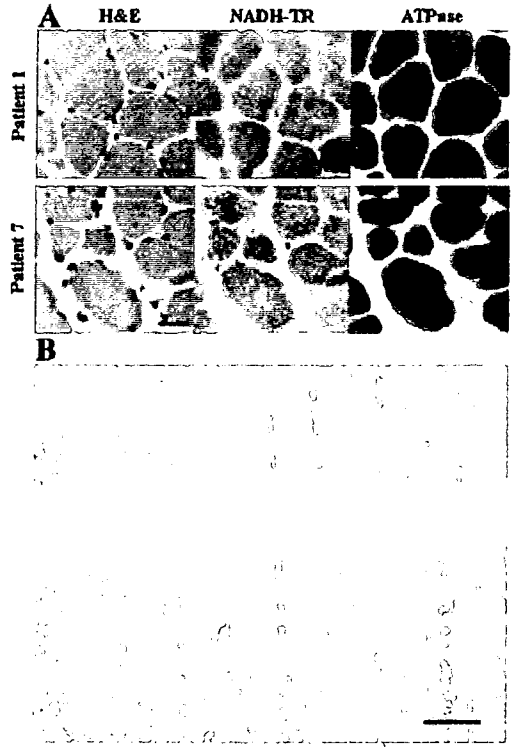
Among patients with and without mutations, there was no difference in the presence of muscle weakness, delayed motor milestones, or skeletal deformity.

**Pathologic findings.** The mean age of biopsy between patients with and without mutations (3.2 ± 2.3, n = 4; 3.2 ± 4.9 years, n = 6) did not differ; all patients showed fiber size variation, regardless of the *RYR1* mutations (table; figure 2). Endomysial fibrosis was mild except in two patients without *RYR1* mutations (Patients 7 and 8). There were no necrotic or regenerating fibers, although a small number of fibers with internally placed nuclei were seen. No group atrophy was noted. No nemaline bodies, ragged-red fibers, or rimmed vacuoles were seen. Intermyofibrillar network was well organized in all fibers without any core or core-like structure. Type 1 fibers comprise more than 99% of fibers. A small number of type 2 fibers were seen except in two patients (Patients 3 and 4), even though the percentage was less than 1%. These type 2 fibers were either type 2B or 2C, and no type 2A fibers were observed.

On electron microscopy, none of 50 fibers observed showed either loss of mitochondria or disorganized myofibrillar structure such as Z-line streaming.

**DISCUSSION** This is the first genetic study for CNMDUI. In 4 of 10 patients (40%), we identified a heterozygous mutation in the C-terminal domain of the gene encoding RYR1, which is vir-

Figure 2 Muscle biopsy




(A) Histochemistry. Patient 1 is a boy age 3 years 5 months (upper panel) with a mutation in *RYR1*. There is mild endomysial fibrosis. Patient 7 is an 8-month-old boy (lower panel) without *RYR1* mutation. Marked fibrosis is observed. In both patients, pathologic findings show marked variation in fiber size, well-organized intermyofibrillar network, and with all fibers composed of type 1 on myosin ATPase staining at pH 4.2. Bar = 20 μm. (B) Electron micrograph. Neither Z streaming nor loss of mitochondria is seen. Bar = 5 μm.

tually exclusively expressed in the skeletal muscle, forming the homotetrameric structures in the sarcoplasmic reticulum membrane, and functions as a $Ca^{2+}$ release channel.[18] As *RYR1* mutations have been associated with three different diseases (CCD, multiminicore disease, congenital myopathy with cores and rods) and MH,[19-22] therefore, CNMDU1 may be the fifth disease linked to *RYR1* mutations.

Among four mutations that we identified, c.14582G→A (p.R4861H) was previously associated with CCD in Europeans,[11] and the c.14761_14762TT→AC (p.F4921T) was previously reported in the father of Patient 4, who had CCD.[11] Two other mutations, c.13013_13032del20 (p.A4338fs) and c.14680G→C (p.A4894P), were novel ones. The 20-bp deletion mutation is predicted to cause a frame-shift, leading to a premature stop codon and removal of the C-terminal 464 amino acid residues from the protein.

The predicted transmembrane helices have been described as M1 to M10.[23,24] However, the recent in vitro study suggested that M1 to M4 regions are actually located in the cytosol and that only M5 to M10 are the transmembrane domains.[25] According to this model, the deletion mutation identified in Patient 1, which was predictably located between M3 and M4, should truncate the protein after the M3 region, losing all transmembrane domains. Furthermore, the previous study showed that the mutant *RYR1* truncated after M3 region can still exist in the cytosol even without being anchored to the membrane.[25] Indeed, the truncated RYR1 protein was present in the patient's muscle as confirmed in Western blot analyses. Our results raise a possibility that the truncated *RYR1* mutant may somehow be associated with the wild-type *RYR1* and disrupt its function. However, the limited amount of the sample did not allow further investigation to clarify the interaction between wild-type and mutant *RYR1*.

Interestingly, c.14680G→C (p.A4894P) affects the same nucleotide and amino acid site with c.14680G→A (p.A4894T), which was found in the MH patient in our previous study.[13] Pathologically, the MH patient with p.A4894T had a normal mosaic pattern of fiber type distribution and not uniform type 1 fiber. Core-like structure was observed in only a few fibers. Proline differs from other amino acids in its structure of imino acid; that is, the side chain of proline forms a cyclic structure.[26] Therefore, a single amino acid change from alanine to proline may lead to a different structural and thereby functional change in RYR1 from that in p.A4894T, resulting in uniform type 1. It is an interesting issue as to whether p.A4894P mutation is also associated with MH. However, no sample was available for in vitro contracture[27] or calcium-induced calcium release test[28] in Patient 3.

We did not find any *RYR1* mutation in six patients in our cohort, suggesting the presence of another causative gene for CNMDU1 and the genetic heterogeneity of the disease, even though there still remains a possibility that mutations may exist in unexamined regions such as the majority of introns. We did not find any *RYR1* mutation in two patients having congenital myopathy with marked type 1 fiber predominance in which type 1 fibers account for less than 99%, suggesting that the *RYR1* mutation in the C-terminal domain may be tightly associated with uniform type 1 fiber, namely, >99% type 1 fibers, albeit a greater number of

patients are needed to make a definite conclusion.

We could amplify only two exons in the C-terminal domain in DNA of the patients first reported to have CNMDU1.[1] Although the original patients were clinically similar to our patients with *RYR1* mutation, in terms of early onset, mild muscle weakness, delayed motor milestones, and pathologic features, their age at the time of biopsy (ages 9 and 12) was higher in comparison with our patients, raising the possibility that the original patients may have had a genetically distinct disorder.

Excitation–contraction (EC) uncoupling caused by *RYR1* mutation is thought to be closely associated with CCD.[29] In vitro studies have shown that two RYR1-binding proteins, FKBP1A and CACNA1S, directly participate or modulate EC coupling in skeletal muscle.[30,31] In addition, 1% of MH patients have mutations in the RYR1-binding region in CACNA1S.[32] Therefore, we sequenced *FKBP1A* and *CACNA1S*, but we did not find a mutation in any patient, suggesting that these genes may not or only rarely be associated with CNMDU1.

In our study, CNMDU1 patients with *RYR1* mutations have mild clinical features compared with those without mutations, in terms of poor fetal movement, asphyxia, infantile hypotonia, respiratory distress, mental retardation, myopathic facies, and high arched palate. This supports the idea that CNMDU1 may be genetically heterogeneous. Most remarkably, none of the patients with *RYR1* mutations had mental retardation, whereas five of six patients without *RYR1* mutations had it. Three of five patients had ventricular dilatation or brain atrophy on brain imaging, suggesting that the mental retardation might occur with a perinatal history of asphyxia or another primary abnormality of unknown origin.

Regarding pathologic findings, CNMDU1 patients either with or without mutations in *RYR1* had similar myopathic changes: mild to marked variation in fiber size. The majority of type 2 fibers, albeit few in number, found in our patients were type 2C, indicating that mature type 2 fibers are even fewer. Patients without *RYR1* mutations had more pathologic variation than those with mutations, suggesting that those without mutations might have genetically different causes.

Solely from the clinical features, it is difficult to differentiate between CNMDU1 patients with *RYR1* mutations and CCD patients with C-terminal mutation in *RYR1*. Both groups of patients show muscle weakness and delayed motor milestones. The frequency of asphyxia, mental retardation, myopathic facies, high arched palate, and skeletal deformities is similar. Furthermore, uniform type 1 fiber is a characteristic pathologic finding in both groups.[11] Two mutations of c.14582G→A (p.R4861H) and c.14761_1476-2TT→AC (p.F4921T) were identified in both CNMDU1 and CCD patients, and all the patients showed type 1 fiber uniformity despite the absence or presence of cores. This result suggests that type 1 fiber uniformity is closely associated with C-terminal *RYR1* mutation. Although additional study is required, there still remains a possibility that CNMDU1 and CCD are closely related diseases, regardless of the presence or absence of cores.

In support of this notion, the father of Patient 4 had CCD, while no cores were observed in the patient's sample.[6] A similar family case was also reported: a 4-month-old girl had CNMDU1 in a family with CCD due to p.Y4864C mutation in exon 101 of *RYR1*.[7] In both families, CNMDU1 was identified in younger children, whereas CCD was found in older family members. These findings suggest that the core may be formed later in the course of disease at least in some patients. Alternatively, cores may not be formed in CNMDU1 patients for factors that are yet to be known.

The fact that we were unable to find distinct pathologic changes other than type 1 fiber uniformity can be due to many possibilities. One is that CCD and CNMDU1 may be a part of a spectrum, as mentioned above. Interestingly, in all familial cases including the one previously reported by others, adults had CCD, whereas children showed CNMDU1, suggesting that cores might not be present in their early lives. In fact, age at biopsy in CNMDU1 patients with C-terminal mutations (3.2 ± 2.3 years, n = 4) was more than 1 year lower than that in CCD patients with C-terminal mutations (4.4 ± 3.0 years, n = 14) in our series,[11] although there is a significant overlap between the two age groups. Nevertheless, we have never found a case with muscle pathology falling between CNMDU1 and CCD, that is, uniform type 1 fiber with cores only in a few fibers. In addition, electron microscopic study of our patient (albeit only one was available) did not show any sign of core formation. These observations may cast some doubt on the notion that CNMDU1 and CCD are part of a spectrum.

Another possibility is that CNMDU1 is actually CCD, and the absence of cores in CNMDU1 may be attributed to the site of sampling. This,

however, is less likely because we have sampled a wide range of sites. MRI studies in CCD have confirmed a distinct pattern of muscle involvement, mostly involving muscle of the lower extremities.[33] In our series, even biceps brachii, which is relatively spared based on clinical examination, actually shows core formation (data not published). The third possibility is that CNMDU1 is a distinct entity, and thus the absence of other pathologic findings may not be influenced by the choice of area sampled.

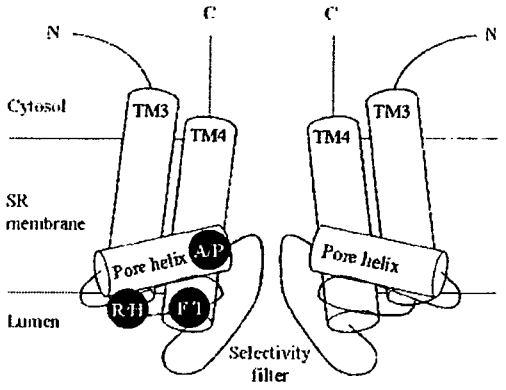
Previously, we have identified 14 CCD patients with heterozygous C-terminal mutation in *RYR1*.[11] Combining this number with that from this study, 4 of 18 patients (22%) with a heterozygous C-terminal mutation are associated with CNMDU1, suggesting that CNMDU1 may not be a rare condition at least among those with the *RYR1* mutation in the C-terminal domain.

The pathomechanism for the development of uniform type 1 fiber is still unknown. Skeletal muscle fiber type formation is thought to be regulated by nerve control and succeeding intracellular signal transduction including $Ca^{2+}$ release that lead to transcriptional activation of fiber type-specific genes. In slow muscle fibers, the lower amplitude and longer duration of $Ca^{2+}$ transition facilitate activation of calcineurin, the $Ca^{2+}$/calmodulin-dependent phosphatase, which dephosphorylates and activates two kinds of transcription factors; nuclear factor of activated T cell (NFAT) and myocyte enhancer factor 2 (MEF2) resulted in significant activation of slow myosin heavy chain 2 gene (slow *MyHC2*) promoter, expressed in avian skeletal muscle.[34-36] In contrast, in fast fibers, high-amplitude calcium sparks induced by infrequent phasic firing of the motor nerves are insufficient to keep activation of calcineurin. When calcineurin is inactivated, phosphorylated NFAT cannot enter the nucleus and the slow fiber-specific program is down-regulated, resulting in the predominant transcription of genes encoding fast fiber-specific proteins.[37]

The inhibition of RYR1 by ryanodine treatment has been reported to induce the activation of slow *MyHC2* promoter in fast muscle fibers via NFAT- and MEF2-dependent transcriptional pathway.[38] It strongly suggests that the loss of function of the RYR1 channel contributes to slow *MyHC2* gene expression. This naturally raises a possibility that the C-terminal mutations found in this study may cause loss of function of RYR1, which leads to the activation of slow fiber-specific program in fast fibers.

In fact, all missense mutations found in CNMDU1 in this study are located in the pore-forming segment of RYR1 (figure 3). This pore-forming segment is located close to the luminal end of the RYR1 channel and is supposed to form the selectivity filter, which plays a critical role in the selection of permeating ions.[39,40] According to this hypothetical model, binding of ryanodine would be expected to change the conformation of this selectivity filter and the inner conduction pore.[41] Therefore, mutations in the pore-forming segment may well alter the interaction of the pore helix and the selectivity filter. Loss of function of RYR1 may explain the mechanism of type 1 fiber uniformity as in the model of RYR1 inhibition by ryanodine, although further investigations are necessary to elucidate the precise pathomechanism for CNMDU1.

**Figure 3** Location of mutation sites in the pore-forming region of RYR1 protein




Putative positions of the three missense mutations (filled circle) of *RYR1* in congenital neuromuscular disease with uniform type 1 fiber found in this study are shown. The putative pore-forming segments from two RYR1 monomers are illustrated as TM3 (outer helix), TM4 (inner helix),[39] same as M8 and M10,[22] and the pore helix connected together.[39] The selectivity filter is indicated. R4861H and A4894P are located at each end of TM3 and the pore helix, whereas F4921T is situated at the beginning of TM4.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