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Central core disease is due to *RYR1* mutations in more than 90% of patients

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Ryanodine receptor 1 (*RYR1*) gene mutations are associated with central core disease (CCD), multiminicore disease (MmD) and malignant hyperthermia (MH), and have been reported to be responsible for 47–67% of patients with CCD and rare cases with MmD. However, to date, the true frequency and distribution of the mutations along the *RYR1* gene have not been determined yet, since mutation screening has been limited to three 'hot spots', with particular attention to the C-terminal region. In this study, 27 unrelated Japanese CCD patients were included. Clinical histories and muscle biopsies were carefully reviewed. We sequenced all the 106 exons encoding RYR1 with their flanking exon–intron boundaries, and identified 20 novel and 3 previously reported heterozygous missense mutations in 25 of the 27 CCD patients (93%), which is a much higher mutation detection rate than that perceived previously. Among them, six were located outside the known 'hot spots'. Sixteen of 27 (59%) CCD patients had mutations in the C-terminal 'hot spot'. Three CCD patients had a probable autosomal recessive disease with two heterozygous mutations. Patients with C-terminal mutations had earlier onset and rather consistent muscle pathology characterized by the presence of distinct cores in almost all type 1 fibres, interstitial fibrosis and type 2 fibre deficiency. In contrast, patients with mutations outside the C-terminal region had milder clinical phenotype and harbour more atypical cores in their muscle fibres. We also sequenced two genes encoding RYR1-associated proteins as candidate causative genes for CCD: the 12 kD FK506-binding protein (*FKBP12*) and the α1 subunit of L-type voltage-dependent calcium channel or dihydropyridine receptor (*CACNA1S*). However, no mutation was found, suggesting that these genes may not, or only rarely, be responsible for CCD. Our results indicate that CCD may be caused by *RYR1* mutations in the majority of patients.

Keywords: central core disease; genotype–phenotype correlation; muscular dystrophy; myopathy; ryanodine receptor 1 mutations

Abbreviations: CACNA1S = α1 subunit of L-type voltage-dependent calcium channel; CCD = central core disease; CICR = calcium-induced calcium release; EC = excitation–contraction; *FKBP12* = FK506-binding protein, 12 kD; mGT = modified Gomori–Trichrome; MmD = multiminicore disease; MH = malignant hyperthermia; *RYR1* = ryanodine receptor 1; SERCA = sarco-endoplasmic reticulum Ca^{2+} ATPase; SR = sarcoplasmic reticulum

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Introduction

Central core disease (CCD) was the first described congenital myopathy in humans (Shy and Magee, 1956), usually inherited in an autosomal dominant pattern, except for few reports on autosomal recessive cases (Manzur *et al.*, 1998; Jungbluth *et al.*, 2002). The clinical features are quite variable (Quinlivan *et al.*, 2003), ranging from lack of visible weakness or abnormality to lack of independent ambulation. Most of the patients, in classical descriptions, have a slowly or

non-progressive proximal muscle weakness and hypotonia during infancy that can persist throughout adolescence/adulthood, and have associated delayed motor development and reduced muscle bulk. In addition, musculoskeletal alterations including congenital hip dislocation, kyphoscoliosis and joint contractures are also common findings in CCD patients (Lorenzon *et al.*, 2000; Quinlivan *et al.*, 2003). The diagnosis is commonly made from muscle biopsy by the presence of cores in type 1 fibres, which are typically well demarcated and located centrally in the fibres. A few cores, however, can be located in the subsarcolemmal regions of individual type 1 muscle fibres. Longitudinal sections show that the core runs the whole length of the fibre.

Recent reports have documented that the phenotypic presentation varies considerably from no visible disability to lack of independent ambulation. While the overall incidence of CCD is rare, the absence of symptoms in a significant number of patients may suggest that the actual incidence of CCD may be considerably higher than that perceived currently.

CCD has been linked to the gene encoding the skeletal muscle ryanodine receptor, *RYR1*, and is considered to be an allelic disease of malignant hyperthermia (MH) susceptibility. This is a pharmacogenetic disorder with autosomal dominant inheritance in which susceptible individuals develop generalized muscle contracture followed by a hypermetabolic state due to massive calcium release from the sarcoplasmic reticulum (SR), when they are exposed to inhaled general anaesthetics or to the depolarizing muscle relaxant succinylcholine. CCD patients have higher probability to be susceptible to MH. The *RYR1* mutations linked to MH and CCD are clustered in three relatively restricted regions of the protein or 'hot spots': N-terminal (residues p.M1-R614), central (p.R2163–p.R2458) and C-terminal (p.R4136-p.P4973). They are also called domains 1–3, respectively (Treves *et al.*, 2005). The first two domains are located in the soluble cytoplasmic regions of the protein. Most of the CCD mutations are clustered in domain 3, which is located in the C-terminus and comprises the transmembrane/luminal and pore-forming region of the channel (McCarthy *et al.*, 2000; Monnier *et al.*, 2001; Davis *et al.*, 2003). Hence, mutations in this region of the protein may directly alter the permeation/selectivity/gating properties of the channel (Balshaw *et al.*, 1999).

The *RYR1* is one of the largest described genes in humans, spanning >159 kb in size on chromosome 19.q13.1. A 15 117-nucleotide-long open reading frame encoded in 106 exons (two of which are alternatively spliced) produces a 563 kD protein. In addition, it forms a homotetrameric structure that functions as an SR calcium-release channel regulating Ca^{2+} content in skeletal muscle during excitation–contraction (EC) coupling. Because of the size of the *RYR1* gene, efficient routine screening for mutations has been difficult. Most of the *RYR1* mutation screenings in CCD patients have been limited to the above-mentioned three 'hot spots' or even to the C-terminal region alone, and 47–67% patients were found carrying *RYR1* mutations (Monnier *et al.*, 2001; Davis *et al.*, 2003; Shepherd *et al.*, 2004), which suggested that CCD is a genetically heterogeneous disease. Here, we screened all the exons and flanking exon–intron boundaries of *RYR1* in order to determine the frequency and distribution of mutations, and describe the genotype–phenotype correlation in Japanese patients with CCD.

Although no mutation in other genes has been associated with CCD, many studies have shown that mutants of the RYR1-associated proteins FKBP12 and CACNA1S cause EC uncoupling *in vitro* just as some *RYR1* mutants do (Avila *et al.*, 2003*b*; Lyfenko *et al.*, 2004; Weiss *et al.*, 2004), raising a possibility that *FKBP12* and/or *CACNA1S* mutations may also be responsible for CCD. In addition, the mutations in the *RYR1*-binding domain of *CACNA1S* are thought to account for 1% of MH patients (Stewart *et al.*, 2001). We therefore sequenced the entire open reading frame of *FKBP12* and the part of *CACNA1S* that encodes the *RYR1*-binding region.

Methods

Subjects

Unrelated Japanese CCD patients were selected for the study from the *National Center of Neurology and Psychiatry (NCNP)* database from 1982 to 2004. CCD diagnosis was established on the basis of characteristic muscle pathology findings of cores almost exclusively in type 1 fibres. We excluded multiminicore disease (MmD) cases, which had multiple cores in >70% of type 1 fibres in our series. Available blood samples from the patients' relatives were also included in the analysis. In addition, DNA samples from 150 subjects without any known muscle disease were studied. Informed consent was obtained from the patients or their parents, as well as from control subjects.

The patients' clinical features were assessed by careful review of their medical records. Pathological features of all patients were independently evaluated by four authors (S.W., M.C.V.M., I.N. and I.N.). All patients have undergone a battery of histochemical stains, including haematoxylin and eosin, modified Gomori–trichrome (mGT), NADH-tetrazolium reductase (NADH-TR) and myosin ATPase.

In order to evaluate the genotype–phenotype correlation in CCD patients, the patients were divided into four groups, respectively, provided that they had one heterozygous C-terminal mutation, one heterozygous non-C-terminal mutation, two heterozygous mutations or no mutation.

Data were entered in Statistics Software for Social Sciences (SPSS version 11.0). Demographic characteristics were analysed by computation of the frequency, the mean ± standard deviation (SD), or the mean ± standard error of means (SEM), whichever was appropriate. The data then were subjected to a univariate analysis (Fisher's exact test). For comparing age and type 2 fibre deficiency, Mann–Whitney test was employed.

Mutational analysis

Genomic DNA was extracted from either muscle biopsy samples or peripheral blood lymphocytes according to standard protocols (Sambrook *et al.*, 2001). PCR primers were designed to amplify all 106 exons of *RYR1*, all five exons of *FKBP12* and the seven

exons of *CACNA1S* (exon 14–17 and 25–27) that encode the RYR1-interacting region (Peng *et al.*, 1998). Amplified fragments were directly sequenced using BigDye Terminator® v3.1 Cycle Sequencing kits on ABI3100 automated Genetic Analyzer (Applied Biosystems™, USA). DNA sequences were analysed with the SeqScape program and compared with the reference genomic sequence of: *RYR1* (Genbank J05200), *FKBP12* (Genbank M92423) and *CACNA1S* (Genbank L33798).

Results

Patients

A total of 27 unrelated Japanese CCD patients were selected for the study, consisting of 7 males and 20 females (Table 1) ranging in age from 2 to 63 years [29 ± 19 years (mean ± SD)] at the time of muscle biopsy. DNA from the parents of two patients (Patients 23 and 25) were also studied.

Patients 1–8 underwent muscle biopsy because they had pertinent family history of MH and were considered to be MH susceptible. In Japan, the diagnosis of MH susceptibility is achieved by detecting enhancement in the rate of calcium-induced calcium release (CICR) from the sarcoplasmic reticulum in chemically skinned muscle fibres (Ibarra *et al.*, 2005), reflecting the underlying pathomechanism of this disorder, that is, a lower activation threshold of the SR calcium-release channel. Cores were incidentally found in the muscle biopsies of Patients 1–8; thus a working diagnosis of CCD was made despite the absence of any muscle symptoms in most of these patients. CICR rate was enhanced in all eight patients. There was no description about the MH susceptibility status on the medical records of other subjects (Patients 9–27).

Nemaline bodies were identified in Patients 11, 12 and 23 on mGT stain; hence they were labelled as having core/rod disease (CRD).

Mutations

A total of 23 different missense mutations affecting 19 residues in *RYR1* were identified, consisting of 3 previously reported and 20 newly identified missense mutations in 93% of CCD patients (Tables 1 and 2). Two patients did not have any mutation in *RYR1*. All mutations were heterozygous single nucleotide changes, except for the substitution of two consecutive nucleotides (c.14761_14762TT>AC) in Patient 21, which was predicted to result in a single amino acid change (p.F4921T). There were two common heterozygous *RYR1* mutations in this cohort: c.14581C>T (p.R4861C) and c.7522C>G (p.R2508C), identified in 4 out of 25 (16%) and in 3 out of 25 (12%) patients, respectively. No mutations were found in either *FKBP12* or *CACNA1S*.

Twenty-two of 25 (88%) had only one heterozygous mutation: 14 (56%) with a heterozygous C-terminal mutation and 8 (32%) with a heterozygous non-C-terminal mutation. The remaining three (12%) patients had two heterozygous mutations (Patients 23, 24 and 25). Only the parents of Patients 23 and 25 were screened for the respective pair of mutations, as samples were not available from the parents of Patient 24. In Patient 23, the mutation p.D60N was identified in exon 3 of her maternal allele while p.L3606P was found in exon 73 of her paternal allele. In Patient 25, p.E512K in exon 14 was inherited from her mother while p.R4893P in exon 102 was acquired from her father. No muscle samples were available for histological studies from the parents of these two patients. *RYR1* mutations were found in patients with CRD (Patients 11, 12 and 23) (Table 1, Fig. 1). None of the newly identified missense mutations were found in 300 control chromosomes. Eighteen of 19 amino acids predicted to be changed were highly conserved through *RYR1* evolution and most of them were also conserved across the *RYR* species, *RYR1*, *RYR2* and *RYR3* (Table 2).

The mutations found in this cohort of CCD patients are shown in Fig. 2 at their respective location in the *RYR1*, along with those reported previously. In this study, 6 mutations were located outside the mutational 'hot spots', 4 in N-terminal 'hot spot' and 13 in the C-terminal region. These mutations were carried by 8 out of 25 (32%), 4 out of 25 (16%) and 16 out of 25 (59%) patients, respectively. We did not find any mutation in the central 'hot spot' or domain 2 in our cohort.

Genotype–phenotype correlation

Among eight patients with a heterozygous non-C-terminal mutation, two showed mild limb muscle weakness while the others were asymptomatic. The exact age of onset was difficult to ascertain because of the paucity of symptoms. Significant clinical findings during pregnancy and birth were not reported in this group. On muscle histochemistry, type 2 fibre deficiency was seen in only one patient, as he had <1% of type 2 fibres, while these were >12% in others [37 ± 4% (mean ± SD)]. Minimal endomysial fibrosis was found in three patients (37%), while the others had no increase in the interstitial fibrous tissue.

In contrast, limb muscle weakness was present in all patients with C-terminal mutations, and significant symptoms were manifested during the perinatal period (Table 1). There was statistically significant association between the presence of mutation in the C-terminal domain and clinical phenotypic characteristic in the following categories: limb muscle weakness, poor foetal movement during pregnancy, presence of joint dislocation and delayed motor milestone (Fig. 3A). Endomysial fibrosis and type 2 fibre deficiency were observed in most, if not all, patients with C-terminal mutations (Fig. 3B).

In terms of core structure, most patients had single cores (95% of fibres), but multiple cores were also seen especially in patients with heterozygous non-C-terminal mutations. In mutations outside domain 3, most cores were located in the periphery or subsarcolemmal areas (71%). In patients with C-terminal mutations, cores were noted to be

Table 1 Summary of clinical and pathological features of the patients and *RYR1* mutations

Patient	Sex	Pregnancy and birth symptoms	Family history	Onset	Clinical symptoms					CICR	Type 2 fibres†	Type 1 fibres with cores†	Features of cores§					Nemaline bodies	Endomysial fibrosis	RYR1 mutations (Exon)
					Delayed motor milestone	Muscle weakness	Skeletal and/or articular malformation	Others	Age at biopsy (years)				Central cores	Rimmed' cores	Atypical cores	Single core	Multiple cores			
1	F	N	Yes	Ad	No	No	JC		25	E	32	93	15	39	20	83	17	No	Minimal	c.1422G>T, p.Q474H (13)*
2	F	N	Yes	Ad	No	D	Sc	Ptosis	37	E	27	93	22	9	12	96	4	No	No	c.7522C>T, p.R2508C (47)*
3	F	N	No	Ad	No	No			63	E	13	89	22	17	4	92	8	No	No	c.7522C>T, p.R2508C (47)*
4	F	N	No	Ad	No	No	Sc		35	E	13	84	14	11	14	84	16	No	No	c.7522C>T, p.R2508C (47)*
5	F	N	Yes	Ch	No	No	Sc		9	E	40	98	74	12	5	96	4	No	No	c.7522C>G, p.R2508G (47)*
6	M	N	Yes	Ad	No	No			34	E	65	78	14	39	10	86	14	No	No	c.7523G>A, p.R2508H (47)*
7	F	N	No	Ch	No	P			8	E	<1	39	28	9	12	89	11	No	Minimal	c.7635G>C, p.E2545D (48)*
8	M	N	Yes	Ad	No	No			48	E	75	24	43	8	27	99	1	No	Minimal	c.10100A>G, p.K3367R (67)*
9	F	N	No	Ch	Yes	P			4	ND	<1	99	34	75	1	>99	1	No	Moderate	**c.13703T>C, p.L4568P (94)***
10	F	PFM, FI, RI, PS	No	Bi	Yes	P	JD, JC		3	ND	1	99	78	62	1	100	0	No	Marked	**c.13891T>A, p.Y4631N (95)***
11	M	FI	No	In	Yes	G	JD, JC		3	ND	2	68	41	71	28	100	0	Yes	Moderate	**c.13900G>A, p.E4634K (95)***
12	F	PFM, FI	Yes	Bi	Yes	G	JD, JC		2	ND	1	98	12	88	1	100	0	Yes	Minimal	**c.13912G>A, p.G4638S (95)***
13	F	PFM	No	Ch	Yes	P	JC, Sc	FMI, HAP	3	ND	<1	86	80	70	13	98	2	No	Marked	**c.14572A>G, p.N4858D (101)***
14	F	FI	No	In	No	G	JD, JC, Sc		10	ND	1	99	81	74	15	99	1	No	Marked	**c.14581C>T, p.R4861C (101)**
15	M	N	No	Ch	Yes	P			10	ND	<1	99	86	80	1	>98	2	No	Minimal	**c.14581C>T, p.R4861C (101)**

16	F	RI, PS	No	Bi	Yes	P	JD, JC, Sc, Lo		2	ND	3	60	69	78	3	98	2	No	Marked	**c.14581C>T, p.R4861C (101)**
17	F	PFM	No	Ch	Yes	G	JD, Sc		2	ND	<1	98	58	76	5	99	1	No	Marked	**c.14581C>T, p.R4861C (101)**
18	M	N	No	Ch	Yes	P	JC, Lo		3	ND	<1	99	67	80	2	99	1	No	Minimal	**c.14582G>A, p.R4861H (101)**
19	F	PFM, FI, RI	No	Bi	Yes	G	JD, Sc	FMI	2	ND	<1	83	21	55	36	100	0	No	Minimal	**c.14693T>C, p.I4898T (102)***
20	F	PFM, FI	No	Ch	Yes	P			9	ND	<1	96	97	72	4	99	1	No	Minimal	**c.14759C>A, p.T4920N (102)***
21	M	N	Yes	Ch	No	P	Lo		6	ND	<1	>99	74	86	6	99	1	No	Minimal	**c.14761_14762TT>AC, p.F4921T (102)***
22	M	PFM, FI, RI	No	Bi	Yes	P	JD, Sc	FMI	3	ND	<1	99	34	82	23	82	18	No	Marked	c.14762T>C, p.F4921S (102)*
23	F	FI	Yes	In	Yes	P	Lo		5	ND	9	66	12	12	30	98	2	Yes	Moderate	c.178G>A, p.D60N (3); c.10817T>C, p.L3606P (73)*
24	F	FI, RI, PS	No	Bi	Yes	G	JD, Lo		10	ND	<1	14	58	62	9	99	1	No	No	c.1280C>T, p.S427L (13); **c.14696G>A, p.G4899E (102)***
25	F	FI, RI, PS	No	Bi	Yes	P	JD, Lo		2	ND	21	>99	89	66	29	98	2	No	Moderate	c.1534G>A, p.E512K (14); **c.14678G>C, p.R4893P (102)***
26	F	N	Yes	Ch	Yes	P	Sc		36	ND	13	99	94	0	74[¶]	98	2	No	Minimal	Not detected
27	F	N	No	Ad	No	D			35	ND	72	52	60	18	31	100	0	No	None	Not detected

C-terminal mutations are written in bold letters. F = female; M = male; N = normal; Ad = adolescence; Ch = childhood; Bi = birth; In = infancy; PFM = poor foetal movement; FI = floppy infant; RI = respiratory insufficiency; PS = poor sucking; JC = joint contracture, JD = joint dislocation; Sc = scoliosis; Lo = lordosis; FMI = facial muscle involvement; HAP = high-arched palate; D = distal, P = proximal, G = generalized; CICR = calcium-induced calcium release; E = enhanced; ND = not done. * Novel mutations; [§]Values expressed as percentage of cores; [†]Values expressed as percentage of fibres; [¶]Refer to text and/or figure for description of cores.

Table 2 Comparison of amino acid in the affected residues with that from mouse, pig, rabbit and other human RYR proteins, RYR2 and RYR3

Residue	60	427	474	512	2508	2545	3367	3606	4568	4631	4634	4638	4858	4861	4893	4898	4899	4920	4921
Predicted change	N*	L*	H*	K*	G/H/C*	D*	R*	P*	P*	N*	K*	S*	D*	H/C	P*	T	E*	N*	S/T*
RyR1																			
Human	D	S	Q	E	R	E	K	L	L	Y	E	G	N	R	R	I	G	T	F
Mouse	D	S	Q	E	R	E	K	L	L	Y	E	G	N	R	R	I	G	T	F
Pig	D	S	Q	E	R	E	K	L	L	Y	E	G	N	R	R	I	G	T	F
Rabbit	D	S	Q	E	R	E	K	–	L	Y	E	G	N	R	R	I	G	T	F
RyR2																			
Human	D	K	Q	E	R	D	K	L	L	Y	E	G	N	R	R	I	G	T	F
RyR3																			
Human	D	–	Q	M	R	E	K	V	L	Y	Q	G	N	R	R	I	G	T	F

*Novel mutations.

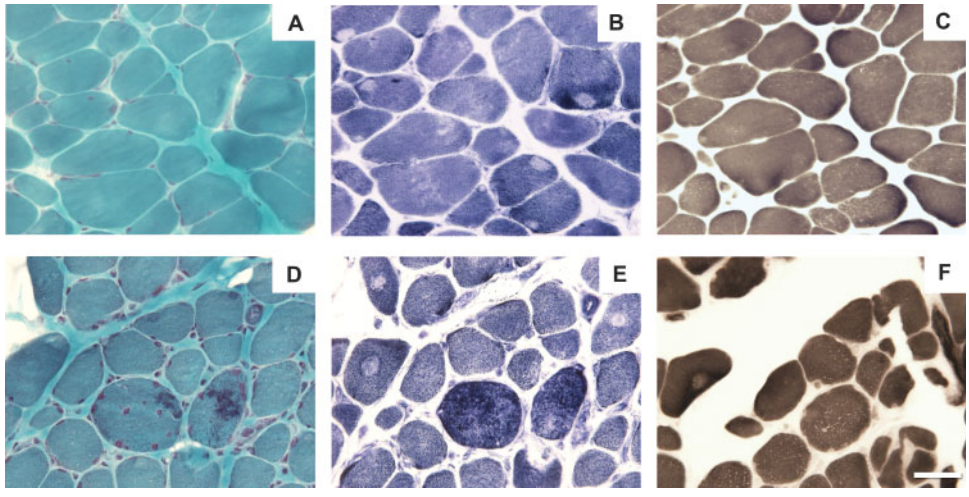

Fig. 1 Muscle biopsy. (**A–C**) A 10-year-old patient with a double mutation. (**A**) Minimal fibrosis is observed in mGT section. (**B**) NADH shows cores in type 1 fibres. (**C**) ATPase staining at pH 4.6 shows type 2 fibre deficiency. (**D–F**) A 3-year-old with nemaline bodies in muscle fibres. (**C**) Nemaline bodies and moderate fibrosis are depicted in mGT section. (**D**) Fibres with nemaline bodies have high enzymatic activity; typical central cores are noted. (**E**) ATPase staining with pH 4.3 pre-incubation demonstrates type 2 fibre deficiency. Bar denotes 50 microns.

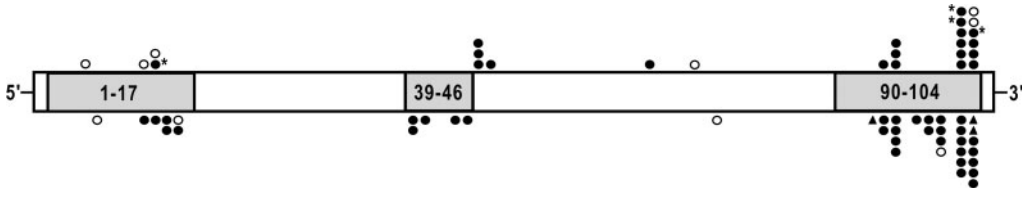

Fig. 2 *RYR1* mutation map for CCD. The three mutational hot spot areas are shaded: CCD domain 1: 1–17; CCD domain 2: 39–46; CCD domain 3: 90–104. Missense mutations (closed circle) found in this study are shown at the top; previously reported mutations are indicated at the bottom. Open circle: recessive mutation; triangle: deletions; asterisk: mutations identified in this study but were also included in previous reports.

characteristic: these were ovoid in shape and with clearly demarcated borders, and were predominantly located in the centre of the fibres [60 ± 7% (mean ± SEM)]; they also occurred singly (98 ± 4%). In addition, most of the cores (72 ± 5%) in this group appeared to be 'rimmed' (Fig. 4B), which may connote high enzymatic activities around the cores on NADH-TR stain. More cores in type 1 fibres were also noted (90 ± 4%), but in general the percentage of the fibres with cores varied from 3 to 100%. No correlation was seen between clinical severity and the percentage of cores in fibres.

Some fibres show 'atypical' cores, characterized by indistinct borders and whose shapes were inexplicitly ovoid (Fig. 4E). These were seen more in non-C-terminal mutations but no statistical significance was observed as compared with C-terminal mutations (Fig. 3B). Like in typical core structures, these were also noted to be either in the centre of the fibre or in the subsarcolemmal areas.

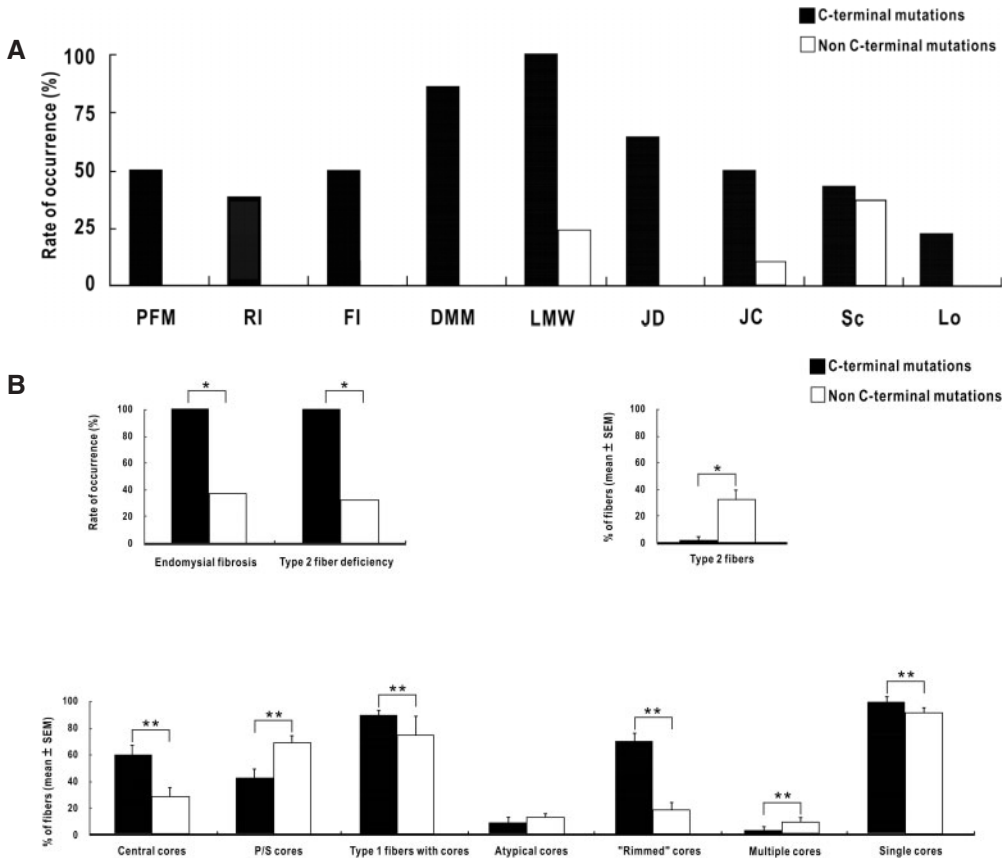

Fig. 3 (**A**) Genotype–phenotype correlation (clinical). Comparison of the clinical features between the patients with a heterozygous C-terminal mutation and those with a heterozygous non-C-terminal mutation. PFM = poor foetal movement; RI = respiratory insufficiency; FI = floppy infant; DMM = delayed motor milestone; JD = joint dislocation; JC = joint contracture; S = scoliosis; L = lordosis; LMW = limb muscle weakness. **P* < 0.05, Fisher's exact test. (**B**) Genotype–phenotype correlation (pathological). Comparison of the pathological features between the patients with a heterozygous C-terminal mutation and those with a heterozygous non-C-terminal mutation. Note the characteristic endomysial fibrosis and fibre type 2 deficiency in patients with C-terminal mutations (upper panel). P/S = peripheral or subsarcolemmal. **P* < 0.05, Fisher's exact test. ***P* < 0.05, Mann–Whitney test; SEM, standard error of means.

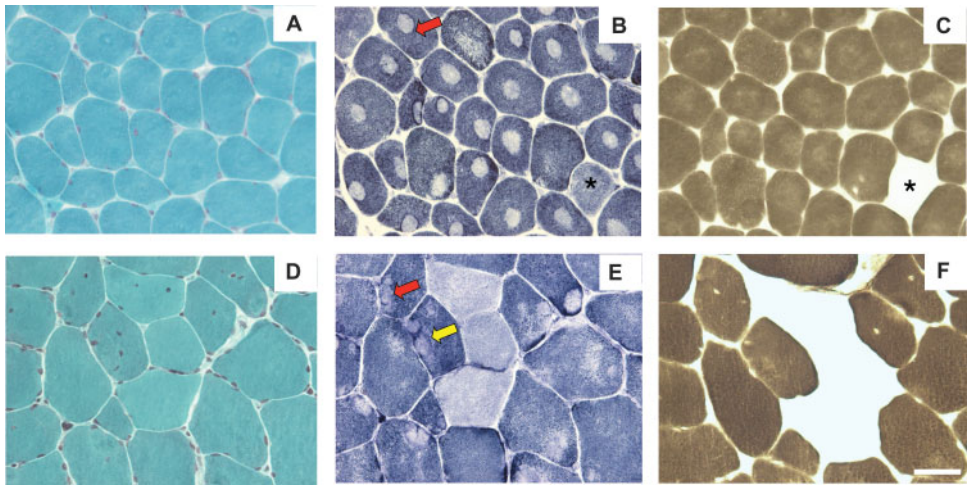

Fig. 4 Muscle biopsy. (**A–C**) A 9-year-old patient with a mutation in C-terminal region. (**A**) Minimal fibrosis is seen in mGT. (**B**) NADH-TR stain reveals the 'central core' in almost all fibres; note the 'rimming' of some cores (arrow). (**C**) Myosin ATPase staining, pH 4.4, clearly demonstrates type 2 fibre deficiency; type 2 fibre is marked with asterisk. (**D–F**) A 63-year-old patient with non-C-terminal mutation. (**D**) Minimal fibrosis is observed in mGT. (**E**) NADH shows cores but not on all type 1 fibres; note atypical cores (red arrow) and multiple cores in fibres (yellow arrow). (**F**) ATPase at pH 4.5 shows type 2 fibres in higher frequency compared with patient with C-terminal mutation. Bar denotes 50 microns.

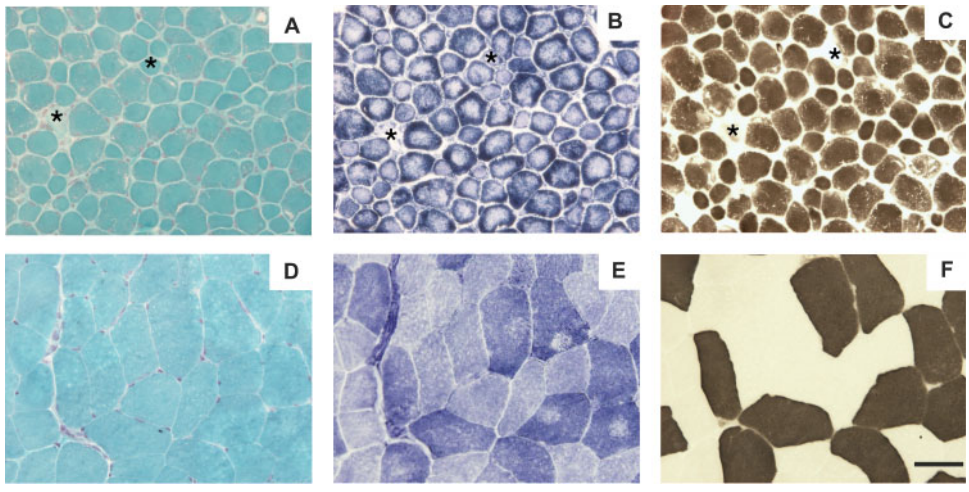

Fig. 5 Sections from patients in whom mutations were not found. (**A**–**C**) Sections from a 36-year-old female. (**A**) Moderate endomysial fibrosis is observed in mGT. (**B**) Cores are seen in almost all fibres; note that cores occupy the whole diameter of fibres, and the subsarcolemmal area surrounding the cores has high oxidative enzyme activity. (**C**) Type 2 fibre (asterisk) deficiency is seen in ATPase with pH 4.4 pre-incubation. (**A**–**C**) Biopsy findings from a 35-year-old female. (**D**) No endomysial fibrosis is noted in mGT. (**E**) Cores were seen but only in few type 1 fibres. (**F**) No fibre type 2 deficiency is observed (type 2 fibres comprised 72%). (**C**) Scale bar denotes 50 microns.

In Patients 1–8, from whom muscle biopsy was performed for CICR and cores were incidentally identified, all had non-C-terminal heterozygous mutations.

The clinical features of patients with two heterozygous mutations were more similar to those of the group with one heterozygous C-terminal mutation. In terms of muscle pathology, type 2 fibre deficiency was likewise seen (Table 2), similar to mutations involving the C-terminal region. This idea may also be buttressed by the fact that among a total of three patients with CRD, two carried a single C-terminal mutation, while the other had a compound heterozygous mutation. In terms of core pathology, however, they resemble that of non-C-terminal mutations: the cores were seen in 14 and 49% of type 1 fibres in Patients 24 and 25, respectively; and the characteristic 'rimming' of cores is less appreciated (Fig. 1).

In two patients in whom no mutation was found, the cores had peculiar features distinguishable from that seen in patients with identifiable mutations. Particularly, in Patient 26, the cores, albeit located centrally, occupied almost the whole diameter of the fibre, and, notably, the subsarcolemmal area around the cores had increased oxidative activity (Fig. 5). The cores in Patient 27 were initially considered to be regular cores but a closer look has shown that almost all the cores had indistinct borders; moreover, only <20% of type 1 fibres have cores (Fig. 5). In such cases, the term 'core-like' may be more appropriate to describe these structures.

Discussion

RYR1 mutations in CCD

At least 44 reported *RYR1* mutations have been associated with CCD, including 39 missense mutations and 5 deletions (Quane *et al.*, 1993; Zhang *et al.*, 1993; Fletcher *et al.*, 1995; Manning *et al.*, 1998; Lynch *et al.*, 1999; Monnier *et al.*, 2000, 2001; Avila and Dirksen, 2001; Tilgen *et al.*, 2001; Ferreiro *et al.*, 2002; Jungbluth *et al.*, 2002; Robinson *et al.*, 2002; Sewry *et al.*, 2002; Avila *et al.*, 2003*a*; Quinlivan *et al.*, 2003; Zorzato *et al.*, 2003; Shepherd *et al.*, 2004). All these mutations are clustered in the three 'hot spots', except for two (Fig. 2). *RYR1* mutations have been reported to be responsible for 47–67% of patients suffering from CCD, implying that the disease is genetically heterogeneous (Vainzof *et al.*, 2000; Monnier *et al.*, 2001). However, neither the true frequency nor the distribution of CCD-causative mutations has been accurately determined to date, since mutation screening has been limited to the three 'hot spots' of the *RYR1*, or even only to the C-terminal region. According to *in vitro* studies, two RYR1-binding proteins, *FKBP12* and *CACNA1S*, directly participate in or modulate EC coupling, while EC uncoupling is thought to be the linch-pin in the pathogenesis of CCD (Avila *et al.*, 2001; Lyfenko *et al.*, 2004). In addition, mutations have been identified in RYR1-binding region of *CACNA1S* in 1% of patients with MH. We therefore regarded *FKBP12* and *CACNA1S* genes as rational candidates for CCD.

We screened 27 Japanese patients with CCD, diagnosed on the basis of histological findings, without putting much emphasis on their clinical presentation as inclusion criteria, and found that *RYR1* mutations occur in 93% (25 out of 27) of CCD patients, which is a much higher rate than that thought previously. The most common CCD mutation in our cohort was c.14581C>T (p.R4861C). Interestingly, the c.14582G>A (p.R4861H) mutation that affects the same amino acid is the most common CCD mutation in European countries, while it was identified in only one patient from our series. Almost all reported patients bearing c.14582G>A had a positive family history, but all patients carrying mutation c.14581C>T were sporadic in this study and also in the

literature (Monnier *et al.*, 2001; Tilgen *et al.*, 2001; Davis *et al.*, 2003), suggesting that the 14 581 nucleotide might be susceptible to change.

To our knowledge, this is the first endeavour to screen the entire coding region of *RYR1* in a cohort of CCD patients. By screening the C-terminal region alone, we would have found *RYR1* mutations only in 59% (16 out of 27) of CCD patients, which is consistent with detection rates in previous reports, missing the two compound heterozygous mutations. Even extending the mutation screening to the three 'hot spots', we would have found *RYR1* mutations in only 67% (18 out of 27), also missing one compound heterozygous mutation. It thus becomes necessary to screen the entire *RYR1* coding region in CCD patients, however impractical it may be owing to the size of the gene. Interestingly, most of mutations outside the 'hot spots' were localized in exons 47 and 48, which neighbour the central region 'hot spot' (exons 39–46). Hence, mutation detection rate in CCD patients could be increased up to 89% by including exons 47 and 48, which may be a more practical alternative than sequencing the entire *RYR1* gene. In addition, *FKBP12* and *CACNA1S* may not be causative genes for CCD or an extremely rare, if they are, since there was no mutation in *FKBP12* or in the RYR1-binding region of *CACNA1S* genes.

We did not find any mutation in two patients in our cohort, suggesting that CCD may still be genetically heterogeneous even though there still remains the possibility that mutations may exist in unexamined regions, such as promoter region and introns, or that we may have overlooked a mutation. Notwithstanding this likelihood of other probabilities, the pathological characteristics of these patients are clearly different from the rest. If these patients were excluded from the analysis solely on the basis of the 'uncharacteristic' core-like structures, that is, if the inclusion criteria used were more stringent, the detection rate of mutations involving the *RYR1* gene will considerably and significantly increase to 100%.

Probable autosomal recessive CCD

CCD was once thought to be inherited solely via an autosomal dominant mechanism, but actually rare instances of recessive inheritance have also been identified (Manzur *et al.*, 1998; Ferreiro *et al.*, 2002; Romero *et al.*, 2003).

In this study, sequencing of the entire *RYR1* coding regions led to the identification of three patients (Patients 23, 24 and 25) with two heterozygous mutations. Patients 24 and 25 needed respiratory mechanical assistance after delivery, while their parents were completely healthy and without any skeletal abnormalities. In both patients, each of the two heterozygous mutations was, respectively, found in each of the parents, confirming that these patients had a compound heterozygous mutation. In Patient 25, p.R4893P affected the third residue of a very well conserved GVRAGGGIGD luminal motif (amino acids p.G4891-D4900) that has been proposed to be a pore-forming fragment responsible for the electrophysiological characteristics of the channel (Zhao *et al.*, 1999).

The parents of Patients 23 and 25 were asymptomatic, while they had heterozygous mutation, suggesting the recessive nature of these mutations. Since CCD patients could be clinically asymptomatic, however, we may not be able to completely exclude the possibility that their parents could have CCD. Nevertheless, judging from the clinical features and the mutation data, autosomal recessive mode of inheritance is most likely in these particular patients. Patient 24 also carried two heterozygous mutations (p.S427L in exon13, p.G4899E in exon 102), but further analysis was inevitably limited by the unavailability of samples from her parents, hampering full evaluation if this was indeed an autosomal recessive case. The p.G4899E mutation also affected the very well conserved GVRAGGGIGD luminal motif (G4899 underlined). Interestingly, this mutation has been reported in two papers as a causative heterozygous mutation (Monnier *et al.*, 2001; Romero *et al.*, 2003); however, since only the 'hot spots' were screened in these studies, the presence of another mutation is still possible and hence it might be impetuous to conclude that p.G4899E mutation is a causative dominant mutation.

Genotype–phenotype correlation

In general, CCD has a wide spectrum of phenotypic expression, ranging from the apparent absence of symptoms to the presence of perinatal complications and generalized muscle weakness. Mutations in the C-terminal region seem to be associated with certain clinical and pathological features: hypotonia during infancy, delayed motor development and limb muscle weakness; type 2 fibre deficiency and interstitial fibrosis; and characteristic cores with clearly demarcated borders, which are observed in almost all type 1 muscle fibres. In addition, 'rimming' on the borders of these cores is observed in much higher frequency; similarly, this phenomenon was also noted in the biopsy specimens of three families determined to have mutation in domain 3 (Sewry *et al.*, 2002). These unique features therefore delineate C-terminal mutations from other groups, at least in terms of muscle pathology.

In contrast, most of CCD patients harbouring at least one mutation outside the C-terminal region had only mild musculoskeletal abnormalities such as joint contractures and scoliosis. This phenomenon may be explained by the leaky-channel model and EC uncoupling model. Some non-C-terminal mutations in *RYR1* promote the leak of Ca^{2+} ions from the SR that may or may not be compensated by the activity of the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), resulting in an elevation of resting cytosolic Ca^{2+} and a depletion of SR Ca^{2+} stores. On the other hand, C-terminal mutations, especially those in the pore region of *RYR1*, may directly affect the channel gating properties, resulting in an abolition of orthograde activation by the

voltage-gated L-type Ca^{2+} channel or, in other words, EC uncoupling. However, there is no compensatory mechanism to increase Ca^{2+} release as SERCA pumps do in the leaky model (Tong *et al.*, 1999; Avila *et al.*, 2001, 2003*b*; Lyfenko *et al.*, 2004). This may explain why C-terminal mutations are associated with clinically evident muscle weakness.

Interestingly, all patients with enhanced CICR had a non-C-terminal heterozygous mutation. Although we did not measure CICR in patients with C-terminal mutations, our results may suggest that majority of CCD patients with MH susceptibility might have a non-C-terminal mutation, considering the facts that no patient with C-terminal mutation had a family history of MH susceptibility, and that one C-terminal mutation, p.I4898T, has been associated with normal halothane and caffeine sensitivities by an *in vitro* study (Lynch *et al.*, 1999). In a recent study made by Monnier *et al.* (2005), where individuals from IVCT-confirmed MH-susceptible families have been screened for *RYR1* mutations and CCD, some MH-normal patients had cores in type 1 fibres albeit the absence of mutation. It would thus be interesting to screen the whole *RYR1* in these patients, as only selected exons were included for genetic screening. In our study, however, we definitely cannot assume that patients with C-terminal mutations may have normal CICR test, as further evaluations are necessary. Furthermore, in only such patients, multiple cores were seen in addition to central cores. Thus, non-C-terminal heterozygous mutations may be more associated with multiple cores and MH susceptibility, although the number of patients is too small to enable us to draw a conclusion. Previous reports have asserted that exclusive MH susceptible patients have mutations in the C-terminal region, but there is only limited study on the histopathological evaluation of these patients, revealing the presence of cores; it is also important to stress that these cores are rather not characteristic of CCD (Ibarra *et al.*, 2006).

We have shown that the muscle pathology on the patients may differ among those with C-terminal mutations and those with mutations outside this area. Cores, moreover, were not only located in the centre of the muscle fibres but also in subsarcolemmal or peripheral areas, and atypical cores can also be seen in such cases, indicating that the possibility of *RYR1* mutation cannot be excluded in patients with such atypical cores. Here, atypical cores of CCD were still different from the cores of MmD, for most of the cores were clearly demarcated and only some fibres appeared to have more than one core; nevertheless, histological, clinical, genetic features and mode of inheritance overlap between CCD and MmD, making the boundary of these two diseases blurred (Lyfenko *et al.*, 2004; Mathews *et al.*, 2004). Patients 24 and 25, who possessed double mutations, had pathology akin to characteristics of both C- and non-C-terminal mutations: type 2 fibre deficiency is seen, and cores are rather atypical and are only seen in moderate number on type 1 fibres. This may, in part, be explained by the fact that these patients carry mutations in and outside the C-terminal region.

In the past, we may have been biased in interpreting the phenotype–genotype correlation between CCD and *RYR1* mutations, since mutation screening had been limited to the 'hot spots'. Quane *et al.* (1993) claimed that various phenotypes could be observed even among patients with the same mutation. However, their patients may have had a compound heterozygous mutation and one of the mutations may have been overlooked. In fact, if we had just screened the *RYR1* C-terminal region of patients with two heterozygous mutations (Patients 24 and 25), we would have found only one mutation, giving an impression of remarkable phenotypic difference between the patients and their healthy parents, regardless of the presence of the same 'heterozygous' mutation.

In summary, our comprehensive mutation screening revealed a surprisingly higher detection rate of *RYR1* mutations in CCD patients than that revealed in previous reports, which accounts for >90% of the cases. Our results also suggest the possibility that many *RYR1* mutations may have been overlooked by the regularly used screening methods that cover only three 'hot spots'. At least, exons 47 and 48 should be included into the hot spot screening if a comprehensive mutation analysis of *RYR1* is not feasible. In terms of muscle pathology, patients with mutations involving the C-terminal region have characteristic cores that can be easily distinguished from those with mutations outside this domain, and also from those patients without mutation in the *RYR1*. Needless to say, the pathomechanism of how these cores develop still remains to be elucidated.

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