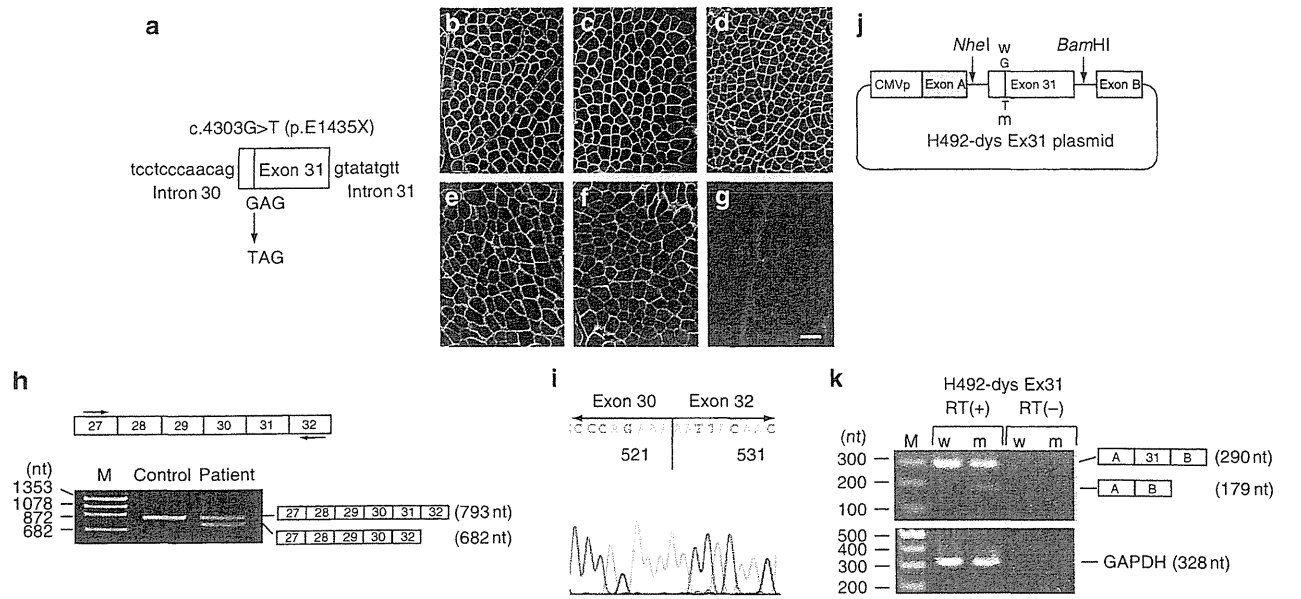

Figure 1 | Point mutation in exon 31 of the *dystrophin* gene causes exon skipping and restores the open reading frame for an internally deleted protein. (**a**) A point mutation that was found in the *dystrophin* gene of patient KUCG797. The position of c.4303G > T (p.Glu1435X) in exon 31 is indicated by the bar. (**b**–**g**) Dystrophin expression in the control (**b**–**d**) and in the patient's (**e**–**g**) muscles. Immunohistochemical examinations for C-terminal (DYS2), N-terminal (DYS3) and rod- (MANDYS1) domains were carried out. Immunoreactivity for DYS2 (exons 77–79 of dystrophin) and DYS3 (exons 10–12 of dystrophin) (**e**, **f**) was somewhat patchy and weaker in the patient's muscles as compared with the control cells (**b**, **c**). However, immunoreactivity for MANDYS1, which recognizes exon 31/32 of the dystrophin was defective only in the patient (compare panels **d**, **g**). Scale bar, 50 μm. (**h**) The RT-PCR products obtained from the control and patient were analysed on agarose gels. The RT-PCR products from the patient's RNA contained an additional shorter product. The DNA sequences of these bands were analysed, and the structure of each PCR product is shown schematically at the right of the panel. (**i**) Sequencing of the shorter product seen in (**c**) confirmed the skipping of exon 31 in the patient. (**j**) A schematic representation of the hybrid minigene plasmids that harbour either the wild-type (W) or mutated (m) exon 31 of the *dystrophin* gene. The minigene vector H492 encodes two cassette exons (A and B) and an intron sequence containing a multicloning site. The *dystrophin* gene region encompassing exon 31 with flanking introns was inserted into the *Nhe*I and *Bam*HI sites in the intron region of H492. These plasmids were transfected into HeLa cells and the pre-mRNAs were transcribed from the cytomegalovirus (CMV) promoter (CMVp). (**k**) The RT-PCR products of wild-type (W) and mutant (m) mRNA that were recovered from transfected HeLa cells were visualized on agarose gel. Two different PCR products were detected only with H492-dys Ex31m plasmid. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control for transfection efficiency. As negative controls, RT-PCR reactions without reverse transcriptase (RT−) were performed for both RNAs from the reporters and GAPDH.

(lanes 3–5 and 8–10). The faster-migrating complex contains one hnRNP A1 molecule on the RNA probe, whereas the slower-migrating complex includes more than two hnRNP A1 molecules[33]. The faster-migrating complex could be detected with both the wild-type and mutant RNA even at a low concentration of GST-hnRNP A1 (lanes 3 and 8), reflecting the efficiency of its formation with either RNA at the hnRNP A1-binding site conserved in exon 31 (ref. 5). In contrast, the slower-migrating complex was formed two times more efficiently with the mutant than with wild-type RNA at each concentration of GST-hnRNP A1 protein (compare lanes 3–5 and 8–10), and the difference was most obvious at the highest protein concentration (0.5 μM; lanes 5 and 10). As the estimated hnRNP A1 protein concentration in the HeLa nucleus is ~145 μM (hnRNP A1 $6–7\times10^7$ molecules per cell[34,35], and 690 μm^3 for the volume of the HeLa cell nucleus[36]), the second binding site of the mutant exon 31 would be occupied by hnRNP A1 *in vivo*. As a control, GST alone was used for the assays and no binding was detected (Fig. 2a, lanes 2 and 7).

Consistent with the low binding score for SRp30c predicted by a Splice Aid analysis, weak binding of the SRp30c protein to the wild-type RNA was detected in the gel mobility shift assay (Supplementary Fig. S3a, lanes 3–5). However, the binding of SRp30c to the mutated RNA was 25–50% lower than that to the wild type (lane 8–10), indicating that the point mutation of exon 31 affects the recognition by SRp30c. All the results described above strongly suggested that the mutated exon 31 was not efficiently recognized as an exon in the context of splicing.

To confirm this, we carried out *in vitro* splicing assays. For the assays, we prepared pre-mRNA that contains either the wild-type or mutant exon 31 in the intronic region of chicken δ-crystallin (CDC) pre-mRNA[37] (Fig. 2b). The production of spliced mRNA containing the wild-type exon 31 (black circle) was detected with CDC-dys Ex31w pre-mRNA after a 60-min incubation (Fig. 2b, lanes 3–5). In contrast, the production of mRNA containing exon 31 from the CDC-dys Ex31m pre-mRNA was suppressed (Fig. 2b, lanes 8–10).

Next we investigated the effect of the overexpression of these RNA-binding proteins on the splicing pattern of a minigene in HeLa cells. As shown in Figure 1f previously, this minigene produced mRNA both with and without exon 31 (Fig. 2c, mock). When SRp30c/SRSF9 was overexpressed, the rate of exon skipping was reduced (Fig. 2c,d, SRp30c/SRSF9). In contrast, overexpression of hnRNP A1 promoted exon skipping (Fig. 2c,d, hnRNP A1, and Supplementary Fig. S4a). Although hnRNP A1 is an abundant protein in HeLa cells, as mentioned previously, we could detect similar amount of the exogenous protein to that of the endogenous protein (Supplementary Fig. S4b). As a control, SRp75/SRSF4, another SR protein[38], was used for the same assay, but failed to change the splicing pattern (Fig. 2c,d, SRp75/SRSF4). These results indicated that the skipping of the exon 31 in the *dystrophin* RNA was promoted

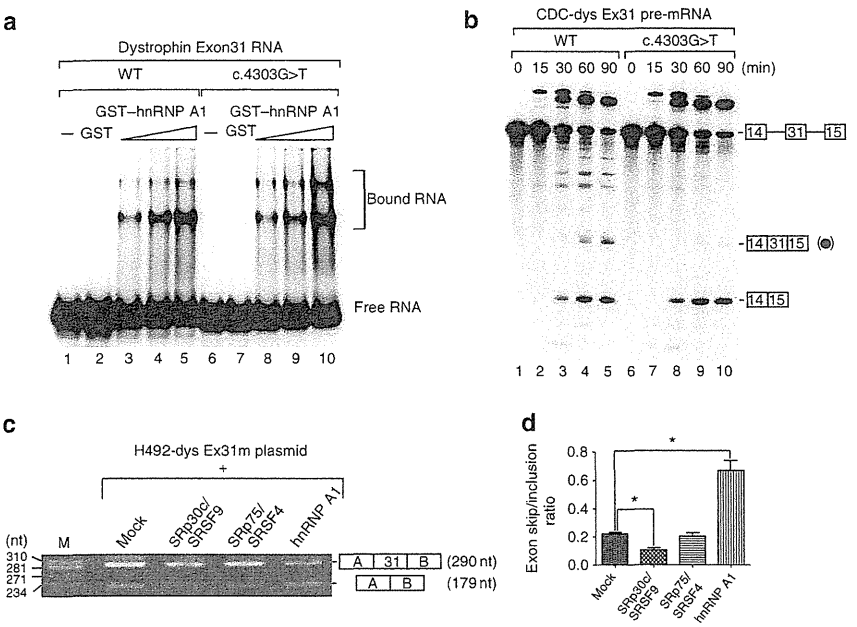

Figure 2 | The point mutation in exon 31 enhances binding to hnRNP A1 and exon skipping both *in vitro* and *in vivo*. (**a**) Gel mobility shift assays with GST-hnRNP A1 and dystrophin exon 31 RNA. ^{32}P-labelled dystrophin exon 31 RNA (wild type or mutant) was incubated with either GST alone (GST: lanes 2 and 7, 400 ng) or with GST-tagged hnRNP A1 (GST-hnRNP A1: lanes 3–5 and 8–10; 100, 200 and 400 ng, respectively), and the resultant complexes were subjected to 8% native polyacrylamide gel electrophoresis. Lanes 1 and 6 show where RNA itself migrates on the gel (marked as Free RNA on the right of the panel). Complexes of hnRNP A1 and RNA are also indicated as Bound RNA. All incubations were carried out at 20 °C for 30 min. (**b**) *In vitro* splicing assays with pre-mRNAs that contain exon 31. ^{32}P-labelled chicken δ-crystallin (CDC) pre-mRNA containing either wild-type (WT) or mutant (c.4303G > T) exon 31 in the intron was incubated with HeLa cell nuclear extracts at 30 °C for the time shown at the top of the panel. The RNA products were analysed by electrophoresis on 6% denaturing gel. The structures of the pre-mRNA and two different mRNAs are shown at the right of the panel. Boxes with numbers indicate exons, whereas the lines between boxes designate introns. The mRNA that includes exon 31 (closed circle) was produced more efficiently with CDC-dys Ex31 WT pre-mRNA than CDC-dys Ex31 c.4303G > T pre-mRNA. (**c**) Effect of overexpression of several RNA-binding proteins on the inclusion and skipping of the mutant exon 31. HeLa cells (3×10^6 cells) were cultured overnight and transfected with 150 ng of the mutant reporter plasmid used in Figure 1f in combination with 1.5 μg of the plasmid that expresses designated proteins with a Flag tag. RNAs were recovered and analysed by RT–PCR. The skipping of the mutated exon 31 was inhibited by SRp30c/SRSF9, but promoted by hnRNP A1. (**d**) Quantification of the ratio between exon skipping and inclusion for the RNA expressed from the mutant reporter plasmid in the presence of several RNA-binding proteins. Averages and standard deviations from three independent experiments are shown. *$P < 0.005$.

by disruption of the SRp30c/SRSF9-dependent ESE and production of an hnRNP A1-dependent ESS in exon 31.

TG003 promotes skipping of a mutated exon 31 in HeLa cells. The fact that this patient can express an internally deleted but presumably partially functional dystrophin protein made us look for chemical compounds that promote the skipping of exon 31 for further production of the internally deleted protein to improve the patient's condition. We screened for specific inhibitors of a variety of kinases and found several compounds that affect alternative splicing[21]. As these chemicals were demonstrated to affect alternative splicing *in vitro* and *in vivo*, we investigated the effect of eight compounds on the mutated exon 31 using the H492 minigene vector (Supplementary Table S2). TG003 strongly induced the skipping of the mutated exon in comparison with SRPIN340 and the solvent alone (Fig. 3a,b, dimethylsulphoxide, TG003 and SRPIN340). To examine the effect of TG003 on the wild-type exon 31, we next incubated cells with different concentrations of the compound after transfection of the minigene plasmids. As shown in Figure 3c, TG003 promoted the skipping of the mutated exon 31 in a dose-dependent manner (Fig. 3c), but did not affect splicing of the wild type even at 50 μM (Fig. 3c). By quantitative RT–PCR analysis, we found that the ratio of skipped mRNA to total spliced mRNAs was increased by TG003 in a dose-dependent manner (Fig. 3d). We then looked for other patients who were sensitive to TG003, and found one patient whose mutated exon was skipped on the administration of TG003 (Supplementary Fig. S5). This dystrophy patient has a point mutation in exon 27 (c.3613delG (p.Glu1205LysfsX9)), which produces a premature termination codon via a frameshift. We cloned the *dystrophin* gene fragment containing the mutant exon 27 and flanking introns into the H492 vector and transfected the plasmid into HeLa cells. As shown in Supplementary Figure S5, exon 27-skipped mRNA production was promoted by TG003 in a dose-dependent manner with the mutant plasmid in HeLa cells, whereas splicing of the wild-type mRNA was not affected.

TG003 promotes *dystrophin* expression in the patient's cells. As the results obtained with a minigene construct strongly suggested that TG003 could induce exon 31-specific skipping, we next examined the effect of TG003 on splicing of the *dystrophin* gene in myoblasts. Muscle cells obtained from the patient were cultured on dishes. Equivalent amounts of dystrophin mRNA with and without exon 31 were detected by RT–PCR (Fig. 4a). When TG003 was added to these cells, it was shown by quantitative RT–PCR analysis that the ratio of exon 31-skipped mRNA to total mRNAs was increased in a concentration-dependent manner (Fig. 4b). To examine the effect of TG003 on the splicing of other *dystrophin* introns, we prepared a subset of primers to amplify all 79 exons of *dystrophin*. The results of RT–PCR with or without TG003 showed that all exons except exon 31 were included (Supplementary Fig. S1 and Supplementary

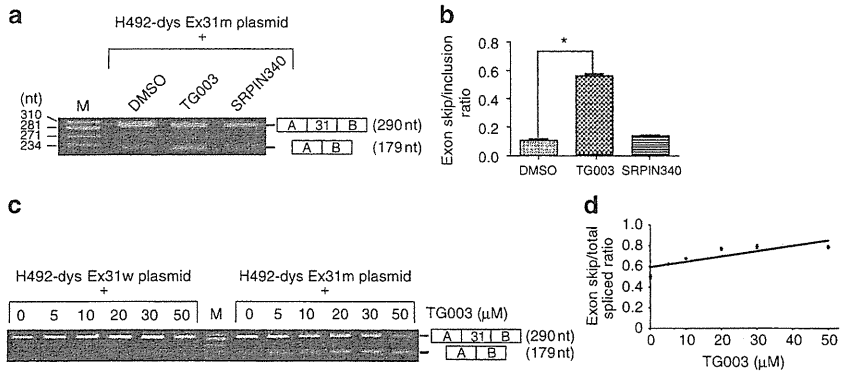

Figure 3 | TG003 promotes skipping of mutated exon 31 in HeLa cells in a dose-dependent manner. (**a**) Effect of chemical compounds on exon 31 skipping with a heterologous reporter construct in HeLa cells. After transfection of the reporter plasmid (H492-dys Ex31m) as given in Figure 2c, HeLa cells were incubated in the presence of the chemical compounds at 30 μM for 24 h. Dimethylsulphoxide (DMSO) was used as a negative control as it was used as a solvent of compounds. RNAs were recovered and analysed by RT-PCR. TG003, but not SRPIN340, promoted skipping of the mutated exon 31. (**b**) Quantification of the ratio between exon skipping and inclusion for the RNA products expressed from the mutant reporter plasmid in the presence of several RNA-binding proteins. Averages and standard deviations from three independent experiments are shown. *$P < 0.0001$. (**c**) RT-PCR of the RNA recovered from the reporter plasmid-transfected HeLa cells cultured in the presence of several concentrations of TG003. (**d**) Quantification of the ratio of exon skipping to total spliced for the RNA products expressed from the mutant reporter plasmid in the presence of several concentrations of TG003 by quantitative RT-PCR. Averages and standard deviations from three independent experiments are shown.

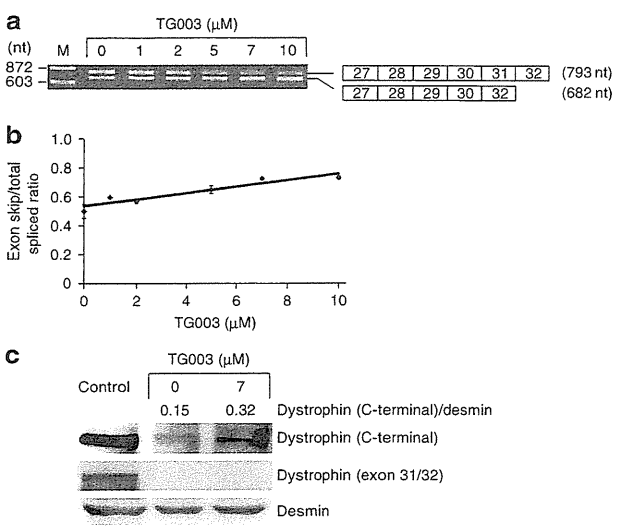

Figure 4 | TG003 induces not only exon 31 skipping but also Δexon 31 dystrophin protein expression in patient cells. (**a**) RT-PCR with RNA recovered from primary cultured muscle cells treated with different amounts of TG003. (**b**) Quantification of the ratio of exon skipping to total spliced for the RNA products expressed from the patient's endogenous *dystrophin* gene by quantitative RT-PCR. Averages and standard deviations from three independent experiments are shown. (**c**) Western blotting of dystrophin protein expression in TG003-treated cells. Proteins were detected with antibodies recognizing either the C terminus of dystrophin or the peptide encoded by exon 31. The antibody against desmin indicated that equivalent numbers of the cells were used for this assay.

Table S1), indicating that TG003 selectively promotes skipping of the mutated exon 31 in the patient's cells (or a mutated exon 27 in another patient).

Therefore, we next examine whether TG003 really increases the expression of the internally deleted, but functional, dystrophin protein in the patient's cells. Western blotting with an antibody that specifically recognizes the C terminus of dystrophin showed that the administration of TG003 (7 μM) increased the protein expression (Fig. 4c). The ratio of dystrophin (C terminal) signal to desmin signal was increased more than double by TG003. The antibody specific for the peptide with exon 31 did not detect it, indicating that the dystrophin protein lacks an exon 31-coding peptide (Fig. 4c). Therefore, TG003 promoted the expression of an internally deleted dystrophin protein by inducing exon 31 skipping in cells derived carrying the c.4303G > T mutation.

Discussion

This paper describes a patient with dystrophinopathy who had a point mutation in exon 31 of the *dystrophin* gene. As the mutation creates an in-frame stop codon, the mRNA was not expected to produce a protein. However, RT-PCR demonstrated that exon 31 was skipped and the dystrophin mRNA was naturally generated in this patient, and an internally deleted dystrophin protein was produced (Fig. 1e–g). We found that the point mutation in exon 31 disrupted the SRp30c-mediated ESE, and simultaneously created an ESS bound to hnRNP A1. This case is similar to that of the *survival of motor neuron 2* (*SMN2*) gene[39–42]. The *SMN2* gene has a C/T transition compared with the *SMN1* gene, which results in prevention of efficient exon 7 splicing. In these cases, either mutations or polymorphisms in exons can affect splicing by disrupting a positive element and creating a negative element[39–42]. In addition to affecting ESE, the c.4303G > T mutation increased the number of hnRNP A1-binding sites in exon 31 of the *dystrophin* gene. There was already one hnRNP A1-binding site in exon 31, but this mutation added another. The hnRNP A1 protein was shown to bind RNA cooperatively[43], and this would be facilitated by two high-affinity binding sites[44]. In our case, the hnRNP A1-cooperative binding seems to be facilitated by the c.4303G > T mutation, affecting the recognition and skipping of exon 31, as shown in Figure 2. It is very likely that there are more diseases and mutant phenotypes caused by the disruption/creation of splicing elements, as alternative splicing is maintained by a balance of positive and negative *cis*-elements in conjunction with trans factors.

Our results demonstrated that TG003 promoted exon skipping only in the mutant exon 31, but not in the wild type. TG003 is a specific inhibitor for Clks[23], and Clks have been shown to phosphorylate SR proteins[24,45–48]. SRp30c/SRSF9 is likely involved in the recognition of exon 31 for splicing (Fig. 2 and Supplementary Figs S2 and S3) and can be a target of TG003. However, TG003 had no effect

on the splicing of the wild-type exon 31, in which SRp30c/SRSF9 is involved (Fig. 3c). We assume that SRp30c contributes to the inclusion of the exon, but it is not phosphorylated by Clk in the nucleus. Therefore, TG003 had no effect on WT exon 31. Moreover, we have currently demonstrated that the overexpression of Clks results in the specific phosphorylation of SRp75/SRSF4 among SR proteins *in vivo*[24]. This means that not all SR proteins are phosphorylated by Clks. It has been reported that the roles of SR proteins in splicing are not always the same. Although it is generally assumed that SR proteins promote the inclusion of exons, some SR proteins can cause the skipping of exons in several alternative splicing events[19,20,49]. Thus, it is possible that some SR protein(s), which is phosphorylated by Clks, remains dephosphorylated by TG003, and this protein cooperates with hnRNP A1 as a coinhibitor of the recognition of exon 31 during splicing. Another possibility is that hnRNP A1 is a specific target of Clks, although there has been no evidence for this. Further experiments are required to examine these possible scenarios.

In this patient, skipping of the mutated exon 31 restores the open reading frame. Therefore, it is likely that an internally deleted, but functional, dystrophin protein is expressed (Fig. 1e–g). This strongly suggests that better clinical features could be expected if the skipping is induced more efficiently to produce more of the internally deleted protein. We found that TG003, reported as a Clk-specific inhibitor, stimulated skipping of the exon in both a minigene construct and the endogenous pre-mRNA in the patient's cells. When we administered 10 μM of TG003 into HeLa and COS cells, no morphological changes were observed and growth rate was not affected up to 3 days[23]. We also confirmed that TG003 had no cytotoxicity in HeLa cells at the dosage used in Figure 3c (data not shown), and even in mice at up to 100 mg kg^{-1} per day *per os* (Onogi H. and Hagiwara M., unpublished data). As shown in Figure 3c, TG003 induced the skipping of the mutated exon 31, but did not affect the splicing of the wild type. The selectivity of the effect of TG003 may explain the weak toxicity of the compound. TG003 was recently shown to inhibit the replication of the influenza virus by modifying the processing of viral RNA in cells[50]. Although more preclinical studies with animal models are needed, TG003 is the first chemical compound that is verified to improve dystrophin production in *in vitro* patient-derived myotubes. We thus started to look for patients sensitive to TG003, and found another dystrophy patient whose mutated exon was skipped (Supplementary Fig. S5). We hope that our findings will contribute towards the development of a therapy of Duchenne muscular dystrophy.

Methods

Case. The proband (KUCG 797) was a 5-year-old boy born to healthy Japanese parents without any family history of muscle disease. He started to walk independently at 1 year and 4 months of age and his motor development was normal. At the age of 2 years, his serum creatine kinase level was found to be 2,567 IU l^{-1} (normal < 169 IU l^{-1}) in a routine blood examination conducted on admission to hospital. He was referred to Kobe University Hospital to have his *dystrophin* gene examined for mutations. The mild creatine kinase elevation persisted (1,331–4,740 IU l^{-1}). No muscle weakness or abnormal gait was observed. At the age of 5 years, a muscle biopsy was performed. Informed consent for all study aspects including genetic testing was obtained from the parents, and these studies were approved by our ethics committees.

Mutation analysis. DNA was isolated from blood samples by standard phenol–chloroform extraction methods. Total RNA was isolated from peripheral lymphocytes that were collected from whole blood using Ficoll–Paque density gradients (Amersham Biosciences AB) or from thin-sliced muscle sections of frozen muscle samples. RT–PCR and RT-nested PCR were used to analyse the dystrophin mRNA expressed in skeletal muscle. A region encompassing exons 27–32 was amplified using an inner set of primers (forward c27f and reverse 2F, Supplementary Table S1). The products were purified and sequenced either directly or after subcloning into the vector pT7 Blue-T (Novagen). The DNA sequences were determined using an automated DNA sequencer (model 310; Applied Biosystems).

Plasmid construction. To construct H492-dys Ex31w and H492-dys Ex31m, the fragments encompassing exon 31 and flanking intronic regions were amplified from the genomic DNA of both the control and the patient by PCR. The primers used were Int 30f-*Nhe*I: 5′-GCGGCTAGCGTGATCCACCTGCCTCGAC-3′ and Int 31r-*Bam*HI: 5′-GCGGGATCCTCAAATCCAATCTTGCCAAT-3′. The amplified products were digested with *Nhe*I and *Bam*HI (New England Biolabs), and inserted into the H492 that had been digested with the same restriction enzymes. For the construction of Flag-SRp30c and Flag-SRp75, PCR-amplified human SRp30c and mouse SRp75 cDNAs were inserted between the *Bam*HI site and *Xho*I site of Flag-pCDNA3 (ref. 51), respectively. Human hnRNP A1 cDNA was amplified by PCR and inserted between the *Bam*HI and *Not*I sites of Flag-pCDNA3 (ref. 52) to construct Flag-hnRNP A1. To construct template plasmids for gel mobility shift assays, PCR-amplified wild-type and mutant *dystrophin* exon 31 were cloned into the *Hind*III and *Xho*I sites of pCDNA3 (Invitrogen). The resultant plasmids were designated as pCDNA3–dys Ex31w and pCDNA3–dys Ex31m. For making pCDC–dys Ex31w and pCDC–dys Ex31m, the wild-type or mutant exon 31 of the *dystrophin* gene was amplified by PCR and inserted between *Sac*I and *Sty*I sites of pCDC[37]. For protein expression in *Escherichia coli*, cDNAs for hnRNP A1 and SRp30c/SRSF9 lacking their termination codons were PCR-amplified and cloned between the *Bam*HI and *Xho*I sites of pET-GSTII[53]. All plasmids described above were verified by sequencing.

Cell culture and transfection. HeLa cells were cultured as described previously[37]. Transfection of the plasmids was carried out by using Lipofectamine 2000 as recommended by the manufacturer. After 24 h of transfection, RNAs were recovered from the transfected cells. The chemical compounds were incubated for 24 h.

Primary culture of the DMD patient's muscle cells. The patient's muscle cells were cultured in DMEM (Sigma) supplemented with 20% fetal bovine serum (Gibco), 4% Ultrocer G (PALL) and 1% antibiotic-antimycotic (Gibco) in a six-well plate (gelatin-coated micro plate (six-well) with lid, IWAKI) until confluent. To induce their differentiation into myotubes, the cells were cultured in DMEM (Sigma) supplemented with 2% horse serum (Gibco) and 1% antibiotic-antimycotic (Gibco) for 2 weeks with or without TG003. The medium and TG003 were refreshed every 2 days.

Immunonohistochemical analyses of skeletal muscle. Skeletal muscle samples obtained from the rectus muscle of the thigh by biopsy were flash-frozen with isopentane cooled in liquid nitrogen. Serial 10-μm-thick frozen sections were analysed with histochemical staining. The procedure for the immunohistochemistry was as follows: serial 10-μm-thick frozen muscle sections were fixed in cold acetone for 5 min. After blocking with normal goat serum, sections were incubated with primary antibodies overnight at 4 °C. The antibodies used were antidystrophin (DYS2: recognizing an epitope in exons 77–79, and DYS3: recognizing an epitope in exons 10–12, Novocastra) and antidystrophin (MANDYS1: recognizing an epitope in exon 31/32, a gift from Professor Dr Glenn E. Morris). After six rinses with PBS, sections were incubated with secondary antibodies, Alexa Fluor 488-labelled goat anti-mouse or anti-rabbit antibodies, at room temperature for 90 min.

Isolation of RNA and RT-PCR. RNA was isolated and analysed by RT–PCR, as described previously[4]. Primers used for amplification of *dystrophin* and human *glyceraldehyde-3-phosphate dehydrogenase* mRNAs are demonstrated in Supplementary Table S1. PCR products were analysed on 2% agarose gels in Tris–borate/EDTA buffer. Skipping efficiencies were determined from gel images by comparing the shortened dystrophin mRNAs to the intact transcript of full length in a densitometric analysis with Image J software (for patient samples) or by quantifying the skipped products with a DNA 1000 LabChip Kit on an Agilent 2100 bioanalyzer (Agilent Technologies; for hDMD mouse samples). Exon skip/inclusion ratios were calculated as the amount of skipped transcript relative to the full-length transcript. Where appropriate, a two-tailed Student's *t*-test was used to determine the statistical significance of the skipping. As DNA size markers, ϕX174-*Hae*III digest (TAKARA) or 2-Log DNA ladder (New England Biolab) was used for agarose gel electrophoresis.

Quantitative real-time RT-PCR. Real-time RT–PCR amplification was performed using a 7500 fast real-time PCR system (Applied Biosystems Inc.). Real-time PCR assays were carried out in a final volume of 20 μl consisting of 10 μl of TaqMan Fast Advanced Master Mix (Applied Biosystems Inc.), 18 pmol each of 5′ and 3′ primer, 5 pmol of specific probe and 10 ng of the sample cDNA. The real-time PCR conditions were as follows: 1 cycle for 20 s at 96 °C, followed by 40 cycles of 2 s at 96 °C for denaturation, 15 s at 60 °C for annealing and 15 s at 72 °C for extension. Spectral data were captured and analysed using 7500 Real-Time Analysis Software version 2.0.3 (Applied Biosystems Inc.). All samples were run in triplicate. β-Actin and desmin was analysed as an endogenous RNA reference gene for normalization of H492-dys 31-derived mRNA and endogenous dystrophin mRNA from the patient, respectively. Exon skip/total ratios were calculated as the proportion of skipped transcript relative to both the full-length and the skipped transcripts.

The PCR primers and probes (5-FAM and 3-MGB) were designed with Primer Express software (Applied Biosystems Inc.), and the sequences of the primers and probes are shown in Supplementary Table S1.

Western blotting with the patient's myotubes. The patient's myotube cells were rinsed twice with PBS and then collected using 1× Cell Lysis Buffer (Cell Signaling Technology). Total protein (60 μg) was loaded on a 3–10% gradient polyacrylamide gel (PAGEL, ATTO). The fractionated proteins were transferred to HYBOND-P (GE Healthcare). Western blotting was performed using the ECL advance Western Blotting Detection kit (GE Healthcare) according to the manufacturer's instructions. The membrane was incubated with an antibody against the C terminus (NCL-DYS2, Leica) and exon 31 (MANDYS8, a gift from Dr Glenn E. Morris) of dystrophin at a dilution of 1:10 and 1:100, respectively. The dystrophin–antidystrophin immune complexes were detected with anti-mouse IgG (GE Healthcare). Western blotting for desmin was performed using the same protocol as described above. The Desmin antibody (H-76, Santa Cruz) was used at a dilution of 1:50. The desmine–antidesmine immune complexes were detected with anti-rabbit IgG (GE Healthcare).

Preparation of recombinant proteins. All proteins were overexpressed in BL21 (DE3) CodonPlus-RIPL cells (Stratagene). GST-His, GST-hnRNP A1-His and GST-SRp30c-His were induced at 20 °C overnight, purified according to the manufacturer's instructions (Pharmacia) and dialysed against PBS. Dialysed proteins were further purified on nickel resin (Qiagen) as recommended by the manufacturer and dialysed against buffer E (20-mM HEPES-KOH pH 7.9, 100-mM KCl, 0.2-mM EDTA, 10% glycerol and 1-mM DTT). Purified proteins were quickly frozen by liquid nitrogen and stored at − 80 °C.

***In vitro* transcription and splicing assay.** As templates, pCDNA3–dys Ex31 and pCDNA3–dys Ex31m were linearized with *Xho*I, and pCDC–dys Ex31w and pCDC–dys Ex31m were linearized with *Sma*I. *In vitro* transcription and purification of the transcribed RNAs were performed as described previously[37]. HeLa cell nuclear extracts were obtained from Cilbiotech. *In vitro* splicing assay was carried out in a 10-μl scale as described previously[37]. The RNAs were analysed by 6% denaturing polyacrylamide gel electrophoresis and autoradiography.

Gel mobility shift assay. Gel mobility shift assays were essentially carried out as described previously[54]. The binding buffer that was used contained 16-mM HEPES-KOH (pH 7.9), 80-mM KCl, 0.16-mM EDTA, 0.8-mM DTT, 8% glycerol, 100 ng μl^{-1} of BSA, 50 ng μl^{-1} of *E. coli* tRNA (Sigma Chemical Co.), 5×10^4 c.p.m. of RNA (dystrophin exon 31 wild type or mutant RNA) and 1 U μl^{-1} of RNasin (Promega). Eight percent native polyacrylamide gels were used to analyse the complexes.

Statistical analyses. Statistical analyses were performed with Prism5 statistical software (GraphPad) using a paired *t*-test or a one-way analysis of variance, followed by Tukey's multiple comparison test.

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Author contributions

A.N., N.K., H.M. and M.M. designed experiments and A.N., N.K., Y.T., M.Y., H.A., M.O. and K.I. performed them. A.N., N.K., H.M. and M.M. analysed the data and wrote the manuscript.

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Brief Communication

Two closely spaced nonsense mutations in the *DMD* gene in a Malaysian family

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ABSTRACT

In Duchenne muscular dystrophy (DMD), identification of one nonsense mutation in the *DMD* gene has been considered an endpoint of genetic diagnosis. Here, we identified two closely spaced nonsense mutations in the *DMD* gene. In a Malaysian DMD patient two nonsense mutations (p.234S>X and p.249Q>X, respectively) were identified within exon 8. The proband's mother carried both mutations on one allele. Multiple mutations may explain the occasional discrepancies between genotype and phenotype in dystrophinopathy.

1. Introduction

The identification of one deleterious mutation in a responsible gene has been considered an endpoint for genetic diagnosis of hereditary diseases. However, multiple mutations have been reported in some genetic diseases. In one survey, the frequency of multiple point mutations causing genetic disease was calculated as ~0.2% (the ratio of the number of multiple point mutations to the total number of reported disease-associated point mutations) [1].

The *DMD* gene is a huge gene comprising of 79 exons locating at Xp21.2. Mutations in the *DMD* gene, exon deletions being the most frequent, result in the severe Duchenne, or the mild Becker, muscular dystrophies (DMD/BMD). Identification of one disrupting mutation in the *DMD* gene is considered sufficient for genetic diagnosis. The reading frame rule explains the clinical differences between DMD and BMD: out-of-frame or nonsense mutations cause dystrophin deficiency, resulting in DMD, while in-frame mutations enable the production of internally deleted dystrophin, resulting in BMD [2]. However, there remain unanswered questions regarding this rule: 1) there are some exceptions in which in-frame mutations result in DMD and *vice versa* [3]; and 2) there can be inter-sibling differences in clinical findings [4]. The answers to these questions might lie in multiple mutations in the *DMD* gene. In the literature, there are no examples of multiple nonsense mutations in the *DMD* gene although a case has been described with a double missense mutation [5].

Here, we identified two closely spaced nonsense mutations in the *DMD* gene during a mutation analysis of Malaysian DMD patients.

2. Methods

2.1. DMD case

The proband was an 18-year-old Malaysian boy with a high serum creatine kinase level (5408 IU/l). He was born to a healthy Malaysian couple as their fifth baby of six. The parents were first-degree cousins. He was diagnosed as having DMD at the age of 4, showing an unstable gait. Genetic diagnosis was performed after obtaining informed consent.

2.2. Methods

Genomic DNA was extracted from blood as described before [6]. Mutations in the *DMD* gene were first screened using multiple ligation-dependent probe amplification (MLPA) using an MLPA DMD kit (SALSA MLPA KIT P034/P035 DMD/Becker; MRC-Holland, Amsterdam, The Netherlands) [7]. If no abnormality was identified by MLPA, four exons (exons 8, 34, 44, and 7) were examined according to Japanese recommendations [8]. The amplified fragments were sequenced directly or after subcloning as described previously [6].

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3. Results

In the proband two nucleotide changes were identified within the 182-bp exon 8: c.701C>A and c.745C>T. The two nucleotide changes were separated by only 44 bp. No nucleotide change was identified in the examined 135-bp intron 7 and 177-bp intron 8. The former would change the TCA serine codon to a TAA stop codon (p.234S>X) and the latter would change the CAG glutamine codon to a TAG stop codon (p.249Q>X). Both were nonsense mutations and were thus concluded to be the cause of DMD.

We questioned whether both mutations were carried by the proband's mother. When we sequenced the exon 8-encompassing region of the mother's genome, we found that she carried both nucleotide changes. Furthermore, subcloning sequencing of PCR-amplified products revealed that one allele was normal and the other carried both of the mutations seen in the proband. We concluded that the two nonsense mutations were inherited through the mother.

Two closely spaced nonsense mutations suggest hypermutability of the inherited allele. However, we did not find any additional nucleotide changes within exon 8, nor in 1340 bp of intron 7 and 1113 bp of intron 8 flanking the exon.

4. Discussion

Two closely spaced nonsense mutations in the *DMD* gene were disclosed in the proband. The two single nucleotide changes were located in exon 8 and were inherited from the patient's mother. To our knowledge, this is the first example of two nonsense mutations identified in the *DMD* gene. C>T changes at CpG sites are considered to arise because of spontaneous deamination of 5-methylcytosine to thymidine at methylated CpG dinucleotides [9], and accordingly, hypermutability of CGA>TGA has been revealed in the *DMD* gene [10]. However, our identified mutations (TCA to TAA and CAG to TAG) were not at CpG sites, indicating that a different mechanism is likely responsible. Moreover, because one mutation is a transition and the other a transversion, it is difficult to envisage an identical mechanism producing both.

Both of these nonsense mutations have been described before. c.701C>A has been identified twice in Australia and c.745C>T has also been revealed twice, in patients from France and the U.S.A. [11]. On each of these four occasions, no additional mutations were described, implying that these nonsense mutations resulted from an isolated mutation event. Multiple mutations can be the result of sequential independent single mutations or they can be generated simultaneously or quasi-simultaneously in the same cell cycle. Multiple synchronous mutations have been postulated to arise *via* transient hypermutability [1]. Multiple mutations are consistent with a model of transient hypermutability [12–14].

Closely spaced multiple mutations are divided into two classes: <100 bp and >100 bp [1]. For the former, closely separated type, which applies to our identified mutations, a so-called 'mutation shower' has been proposed [14]. In an extreme case of mutation shower, five closely separated mutations were identified [1]. The lack of additional mutations in a surrounding region of 2635 bp is slightly more supportive of sequential mutations.

Our findings suggest that unidentified second mutations may play a role in modifying the clinical phenotypes of dystrophinopathy, which has a wide range of clinical severity. Nonsense mutations have occasionally been identified in mild BMD, and exon skipping has been reported to be a modifier in these cases [15–17]. However, some cases with exon deletion do not follow the reading frame rule [8]. Our result suggests that a second mutation could explain a discrepancy between genotype and phenotype that cannot be explained by the reading frame rule. We believe that, although determination of one deleterious mutation in the *DMD* gene is sufficient for genetic diagnosis, it is also necessary to pay attention to additional mutations that may modify the phenotype.

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ORIGINAL ARTICLE

Mutation spectrum of the dystrophin gene in 442 Duchenne/Becker muscular dystrophy cases from one Japanese referral center

Yasuhiro Takeshima[1], Mariko Yagi[1], Yo Okizuka[1], Hiroyuki Awano[1], Zhujun Zhang[1], Yumiko Yamauchi[1], Hisahide Nishio[2] and Masafumi Matsuo[1]

Recent developments in molecular therapies for Duchenne muscular dystrophy (DMD) demand accurate genetic diagnosis, because therapies are mutation specific. The KUCG (Kobe University Clinical Genetics) database for DMD and Becker muscular dystrophy is a hospital-based database comprising 442 cases. Using a combination of complementary DNA (cDNA) and chromosome analysis in addition to conventional genomic DNA-based method, mutation detection was successfully accomplished in all cases, and the largest mutation database of Japanese dystrophinopathy was established. Among 442 cases, deletions and duplications encompassing one or more exons were identified in 270 (61%) and 38 (9%) cases, respectively. Nucleotide changes leading to nonsense mutations or disrupting a splice site were identified in 69 (16%) or 24 (5%) cases, respectively. Small deletion/insertion mutations were identified in 34 (8%) cases. Remarkably, two retrotransposon insertion events were also identified. Dystrophin cDNA analysis successfully revealed novel transcripts with a pseudoexon created by a single-nucleotide change deep within an intron in four cases. X-chromosome abnormalities were identified in two cases. The reading frame rule was upheld for 93% of deletion and 66% of duplication mutation cases. For the application of molecular therapies, induction of exon skipping was deemed the first priority for dystrophinopathy treatment. At one Japanese referral center, the hospital-based mutation database of the dystrophin gene was for the first time established with the highest levels of quality and patient's number.
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Keywords: Becker muscular dystrophy; database; Duchenne muscular dystrophy; dystrophin; mutations

INTRODUCTION

Duchenne muscular dystrophy (DMD; MIM (Online Mendelian Inheritance in Man) no. 310200) and Becker muscular dystrophy (BMD; MIM no. 300376) are allelic X-linked recessive diseases caused by mutations in the dystrophin gene (MIM no. 300377). DMD is the most frequent inherited muscle disease affecting one in every 3500 male births. DMD is characterized by a rapidly progressive disease that is first recognized during childhood; affected individuals commonly lose their ability to walk before they turn 12 years old. In their third decade, they usually succumb because of either cardiac or respiratory failure. BMD has a slower rate of progression; affected individuals remain ambulatory beyond the age of 16 years and may lead near-normal lives. During the infantile period, DMD/BMD can be identified in boys by a high elevation of serum creatine kinase activity, even before muscle weakness manifests.[1]

The dystrophin gene located at Xp21.2 is characterized by its enormous size (2.4 Mb). It consists of 79 exons, forming a 14-kb mRNA transcript, and lengthy introns (up to 250 kb).[2] Mutation studies on the dystrophin gene have focused on detecting deletions or duplications of one or more exons, and multiplex PCR that amplifies selected deletion-prone exons has been used as the most efficient method of mutation detection.[3,4] In these gross rearrangements, the reading frame rule explains the clinical difference between DMD and BMD at the molecular level; that is, deletions or duplications that shift the reading frame of the dystrophin mRNA (out-of-frame) lead to the more severe DMD phenotype, whereas the milder BMD phenotype occurs if the reading frame is preserved (in-frame).[5] This rule has been applicable in >90% of cases.

The recent introduction of the quantitative PCR-based technique of multiplex ligation-dependent probe amplification (MLPA), which amplifies all 79 exons and is commercially available, has significantly improved mutation detection of deletions or duplications.[6–9] However, the identification of small mutations in the dystrophin gene remains challenging, because of the large number of exons and the huge size of the dystrophin gene. By combining quantitative PCR and direct sequencing technologies, the mutation detection rate has risen

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to 98% of DMD cases (106/108 total cases).[10] In spite of technological advances in mutation analysis, a complete mutation spectrum has not been established in a large cohort, leaving some percentage of cases undiagnosed,[11] and the dystrophin gene remain uncharacterized in some percentage of cases.[12]

Identifying a dystrophin mutation in all cases would permit a clinical diagnosis as the basis for proper genetic counseling, and is also a precondition for future molecular therapies, such as correction of the reading frame shift by induction of exon skipping, or suppression of nonsense mutation during translation.[13–15] In Japan, however, there is no large-scale database for mutations of dystrophinopathy. Strategies for molecular therapies or for comprehensive molecular testing remain undefined.

In this study we described the complete mutation spectrum of 422 Japanese DMD/BMD cases and propose strategies for molecular therapies and genetic testing.

MATERIALS AND METHODS

Patients

Japanese DMD/BMD male patients from all of Japan (although mainly from the West) were referred to Kobe University Hospital, Kobe, Japan, for their molecular diagnosis. The KUCG (Kobe University Clinical Genetics) database for dystrophinopathy is the hospital based-database and was completed by identifying mutations in all dystrophinopathy case as of May 2009. This is the largest Japanese dystrophinopathy database, including 530 patients from 442 families. To abolish bias in counting the incidence of mutations, we only included one case per unrelated family in this report. Among 442 cases, 356 or 86 were clinically diagnosed with DMD or BMD, respectively, because of their symptoms or muscle biopsy findings. Extensive mutation analysis of the dystrophin gene was conducted on the supposition that a responsible mutation should be identified in every dystrophinopathy case. In some cases, muscle biopsy was conducted for immunohistochemical examination and dystrophin complementary DNA (cDNA) was analyzed as previously reported.[16–18] Informed consent was obtained for molecular analysis and this study was approved by the ethics committees of Kobe University School of Medicine (approval no. 28 in 1998).

Methods

Our strategies for the detection of mutations in the dystrophin gene changed over time.[16,17,19,20] In brief, genomic DNA (gDNA) was first analyzed to detect deletion or duplication of one or more exons. If no responsible gross mutation was identified, muscle biopsies were obtained for dystrophin immunostaining to confirm the diagnosis.[18] Dystrophin mRNA extracted from lymphocytes or muscle tissue was analyzed using reverse transcription PCR.[16,17]

Analysis of gDNA

Peripheral blood samples were obtained from the patients and their family members. gDNA was isolated using standard phenol–chloroform extraction methods. At the beginning of this study, Southern blot analysis using dystrophin cDNA as a probe was performed with *Hind*III restriction enzyme-digested DNA as a template, as described previously.[21,22] Later, this time-consuming and laborious method was abandoned. In most of cases, multiplex PCR that amplified deletion-prone 19 exons was used for the first line of mutation detection,[3,4] followed by the second-line method of the Southern blotting.[21] When no responsible gross mutation was identified, in some cases all 79 exons were PCR amplified from gDNA and subjected to direct sequencing as previously described.[23,24] In cases in which no responsible mutation could be detected, the dystrophin cDNA was analyzed.[16] As a final step, chromosomal analysis was conducted (Mitsubishi Medience, Tokyo, Japan).

Currently, MLPA (SALSA MLPA KIT P034/035 DMD/Becker; MRC-Holland, Amsterdam, The Netherlands) is our first-line method to identify mutations in the dystrophin gene,[20] because this allows detection of deletions or duplications in all 79 exons. This analysis was conducted by Mitsubishi Medience. In cases with loss of a single exon by MLPA, the exon structure was examined by another method before concluding that there is a single-exon deletion, because small mutations have been found to disturb MLPA amplification.[20]

Analysis of dystrophin mRNA

Reverse transcription PCR was used to analyze the dystrophin mRNA expressed in lymphocytes or skeletal muscle as previously described.[16,25] Full-length dystrophin cDNA was amplified as 10–20 separate, partially overlapping fragments and the amplified products were sequenced. When mutations were identified in cDNA, they were confirmed in gDNA by PCR amplification and direct sequencing of the corresponding genomic region. In cases with deep intron mutations, ambiguous insertions were identified in their cDNA sequence. Subsequently, genomic mutations leading to the pseudoexon inclusion were analyzed by direct sequencing of gDNA.

Sequencing of the amplified products

The amplified products were purified and sequenced either directly or after subcloning into a pT7 blue T vector (Novagen, Madison, WI, USA).[23] The DNA sequence was determined using an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA, USA).

Nucleotide numbers for all the mutations were designated according to the cDNA reference sequence in GenBank, accession number NM_004006.1 (RefSeq NM_004006.1), in which the 'A' of the start codon is nucleotide 1.

Genotype–phenotype correlation

Because the reading frame rule is thought to explain the phenotype,[5] this was examined in all cases. When the exact size of the deletion or duplication was known, the resulting reading frame was designated as out-of-frame or in-frame. For phenotype classification, DMD was diagnosed when cases became wheelchair-bound by the age of 12 years or when dystrophin deficiency was detected on muscle biopsy. BMD was diagnosed when cases could walk unsupported above the age of 13 years.

RESULTS

In this study, all dystrophinopathy cases referred to the Kobe University Hospital for mutation analysis of the dystrophin gene were shown to have a responsible mutation, establishing the KUCG database. Mutations were identified in 442 DMD and BMD cases (Figure 1a), which included 260 different mutations.

Among the 442 mutation events, 270 (61%) and 38 (9%) were large deletions or duplications of one or more exons, respectively. In other words, large deletions and duplications were observed in 308 cases (70%). The ratio of duplication to deletion was 0.14 (38/270), with deletion being seven times more common than duplication. This indicates that deletion and duplication are not reciprocal events. Nonsense mutations were identified in 69 cases (16%). Mutations disrupting the splice site consensus sequences were detected in 24 cases (5%). Small deletions/insertions (one to several hundreds nucleotides) were identified in 34 cases (8%). Interestingly, four deep intron mutations that created pseudoexons with novel splicing consensus sequences were identified in four cases (1%) using dystrophin cDNA analysis. Chromosomal abnormalities were detected in two cases (0.5%). In one BMD case, a splicing abnormality was identified, although no responsible genomic change was evident.[26] It seems that the frequency of these mutations differed depending on the phenotypic group, as shown in Figures 1b and c. A high frequency (67%) of large deletion is observed in BMD cases when compared with DMD cases (60%), whereas nonsense mutations represent only 3% of the mutations in BMD cases when compared with 19% in DMD cases.

Large deletions and duplications

Large deletions of one or more exons were identified in 270 cases (Figure 1). The deletion patterns were categorized into 104 patterns (Figures 2a and b), in which 5 patterns were observed in both DMD

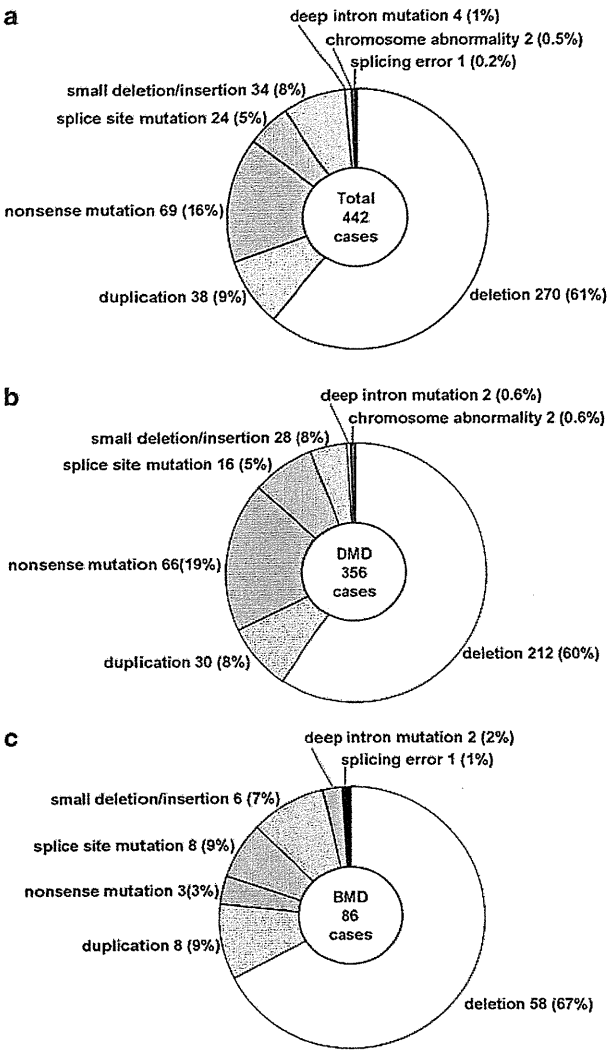

Figure 1 (a–c) Full spectrum of mutations identified in the dystrophin gene among Japanese dystrophinopathy cases. (**a**) Distribution of mutations in 442 dystrophinopathy cases. Gross changes in gDNA were detected by PCR, Southern blotting or multiplex ligation-dependent probe amplification (MLPA) analysis. After this step, deletions and duplications encompassing one or more exons were identified in 270 (61%) and 38 cases (9%), respectively. For the second-line analysis, dystrophin cDNA or exons and intron/exon boundaries of gDNA were sequenced. Nonsense mutations were found in 69 cases (16%). Splice site mutations and small insertion/deletion mutations from 1 to 606 bp long were identified in 24 (5%) and 34 cases (8%), respectively. In four cases (1%), dystrophin cDNA analysis identified novel transcripts with a pseudoexon created by a single nucleotide change deep into an intron. X-chromosome abnormalities were identified in two cases (0.5%). Distribution of mutations in 356 DMD cases (**b**) and 86 BMD cases (**c**).

and BMD (deletions of exons 3–7, 10–44, 43, 44 and 45). The most common pattern was in-frame deletion of exons 45–47, which was found in 12 cases (4.4%); all were BMD (Figure 2b). The second most common pattern was out-of-frame deletion of exons 45–52, 48–50 or the single exon 52; each was found in 11 cases and all of these were DMD (Figure 2a). The largest deletion encompassed exons 1–59, and was found in one case (KUCG no. 513). A single-exon deletion was the most frequently observed pattern, representing 19 patterns among 65 cases. After exon 52 deletion, the second most common single-exon deletion was of exons 44 or 45, both of which were identified in 10 cases (9 DMD and 1 BMD cases with exon 44 deletion, and 8 DMD and 2 BMD cases with exon 45 deletion). Deletions were found to cluster in proximal or distal hotspots (Figure 2). Deletions starting in the distal hotspot (exons 45–55) represented 65% (175/270) of the identified deletions, whereas deletions starting in the proximal hotspot (exons 2–20) accounted for only 26% (69/270). Intron 44 was by far the most frequently involved in deletion breakpoints, preferentially as a starting breakpoint.

Large duplications were identified in 38 cases (9%) and their duplication patterns were categorized into 31 patterns, in which duplication of exon 2 was observed in both DMD and BMD (Figures 3a and b), indicating considerable heterogeneity among the duplication mutations. Out of 31 patterns, 27 were observed only once, whereas 4 were repeated duplication events. Duplication of exon 2 was the most common, observed in four cases (two DMD and two BMD cases). The second most common duplication was that of exons 3–7, found in three DMD cases. Single-exon duplication was observed in six patterns and the largest duplication stretched from exons 3–43. It is noteworthy that the duplicated regions were not in tandem but separated into two regions in two different patterns (the ninth and eleventh lines in Figure 3a).[27] The duplicated exons were distributed across the dystrophin gene and the proximal hotspot was more frequently duplicated than the distal hotspot with deletion mutations (Figures 2 and 3).

Analysis of the breakpoint distribution pattern in the dystrophin gene identified differences between the deletion and duplication mutation events (Figures 2 and 3). Deletion breakpoints were clustered in a few introns in the distal deletion hotspot, whereas they were more disseminated in the proximal region in accordance with the observed higher diversity among duplication events. Exon deletions involved multiple exons with a mean and median of 5.9 and 4 exons, respectively, whereas those for exon duplication were 10.0 and 5 exons, respectively. A greater variability in deletion length was associated with the proximal hotspot, in which more than 10 exons were frequently deleted.

Nonsense mutations

Nonsense mutations represented 16% of all the mutations (69/442) and were the second most common category (Figure 1a). These 62 patterns of single-nucleotide changes were dispersed across the dystrophin gene (Table 1), and 18 were novel (26%). Most were unique but c.5899C>T was found in three cases, and c.2365G>T, c.4729C>T, c.8608C>T, c.9568C>T and c.10108C>T were found in two cases each (Table 1). Among these six repeated nonsense mutations, five, including c.5899C>T, were CGA to TGA mutations. In fact, CGA to TGA transitions comprised nearly a quarter of nonsense mutations (18/69 cases; Table 1). This is because the CpG site is vulnerable to deamination, in which C is replaced by T.[28,29] The CGA codon is found 29 times in the dystrophin gene, and 12 of them (41%) were found to be mutated to TGA in this study. Although nonsense mutations usually cause DMD, three nonsense mutations were identified in the milder, BMD cases (4%) (Table 1). Two were found to produce in-frame dystrophin mRNA with skipping of the nonsense-containing exon,[17,30] and the other was present in the C-terminal region.[31]

Deep intron mutations

In all, four deep intron mutations, located at least 285 bp from an intron/exon junction, were identified (Figure 4). These would not have been identified by conventional gDNA analysis that merely analyzes

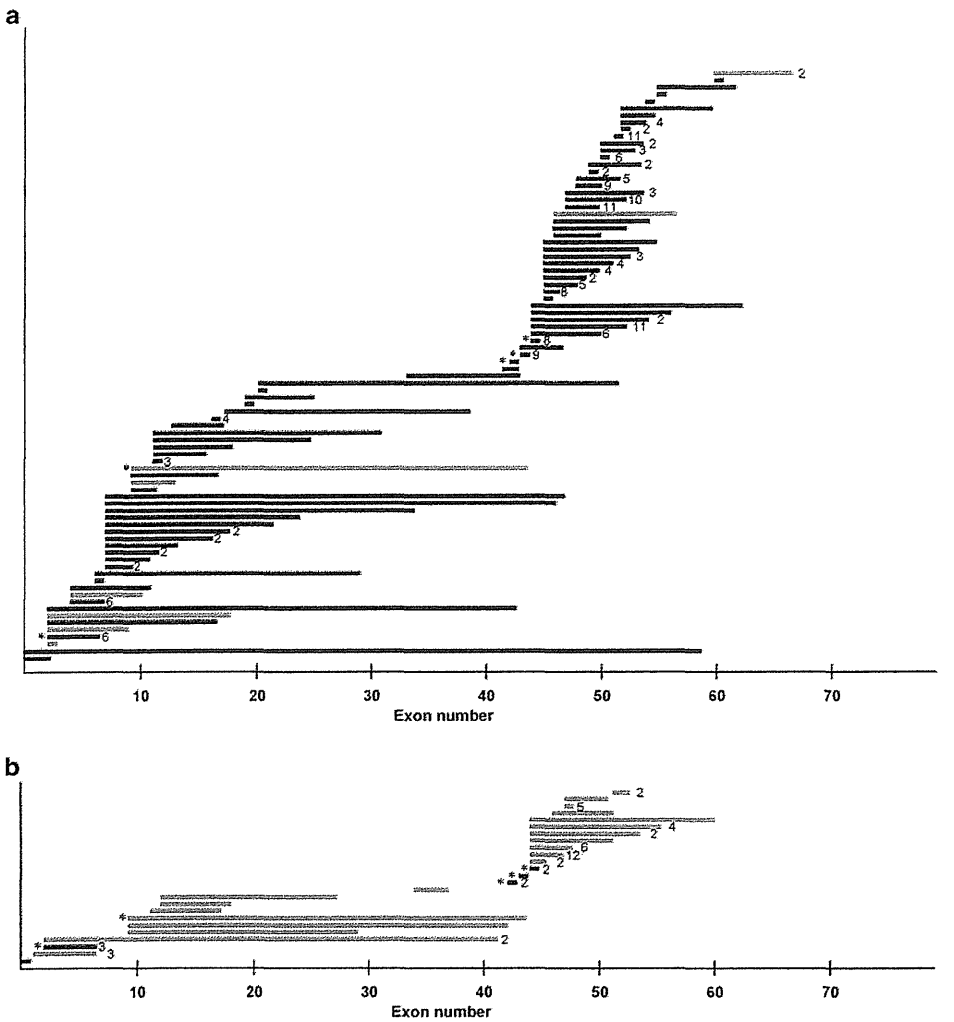

Figure 2 Patterns of exon deletion in DMD (**a**) and BMD cases (**b**). Exon deletions were categorized into 104 patterns. Deleted exon regions are represented by horizontal bars; filled or shaded bars represent out-of-frame or in-frame deletions, respectively. On the bottom line, exons are numbered 1–79. The number at the end of each bar represents the number of cases with an identical deletion, whereas no number means that the deletion is unique. Asterisks indicate the deletion mutations that could result in both DMD and BMD phenotypes. Deletions were clustered in two hotspots: nearly 1/3 were in the proximal region of the gene extending from exons 2 to 20 and the other 2/3 were in the central region extending from exons 45 to 55 in both DMD and BMD cases.

intron/exon boundaries. In these cases, reverse transcription PCR of dystrophin mRNA showed larger amplified products than expected, and sequencing of the products revealed that additional exonic sequence that matched part of an intron was inserted between authentic exons (Figure 4). In one BMD case (KUCG no. 9597), a thymine 5590 bp into intron 2 was replaced with an adenine. This resulted in a splice acceptor consensus sequence and a novel 132-bp exon was included between exons 2 and 3[32] (Figure 4a). In another DMD case (KUCG no. 465), a cytosine 843 bp upstream of exon 28 was replaced with an adenine, producing the consensus splice donor site. Therefore, a novel 119-bp exon was created and incorporated into the dystrophin mRNA (Figure 4b). The other two cases (KUCG nos. 290 and 857) also had single-nucleotide changes deep into an intron 285 or 2714 bp from an intron/exon boundary, respectively, which created splice donor sites. In these cases, pseudoexons (58 and 121 bp long, respectively) were inserted between authentic exons (Figures 4c and d). Insertions of out-of-frame pseudoexons resulted in DMD in KUCG no. 857. However, KUCG no. 290, who also had an insertion of an out-of-frame pseudoexon, showed a BMD phenotype because mRNA without the pseudoexon was also transcribed.

Splice site mutations

Nucleotide changes disrupting the splice site consensus sequence were shown to produce abnormal transcripts in 24 cases (Table 2). Among 24 splice site mutations, 22 were single-nucleotide changes and the other two were a 2-bp insertion and 8-bp deletion, each found in one case. The most common nucleotide change was a G-to-A change at the first intronic nucleotide; this was found in different introns of five cases. A nucleotide change at the first or last nucleotide of an intron was identified in 11 cases, accounting for 46% of all splice site mutations. Mutations at the splice donor site were more common than those at the splice acceptor site (17 vs 7 cases). In this study, no mutations were identified at the branch point. The splicing pathway

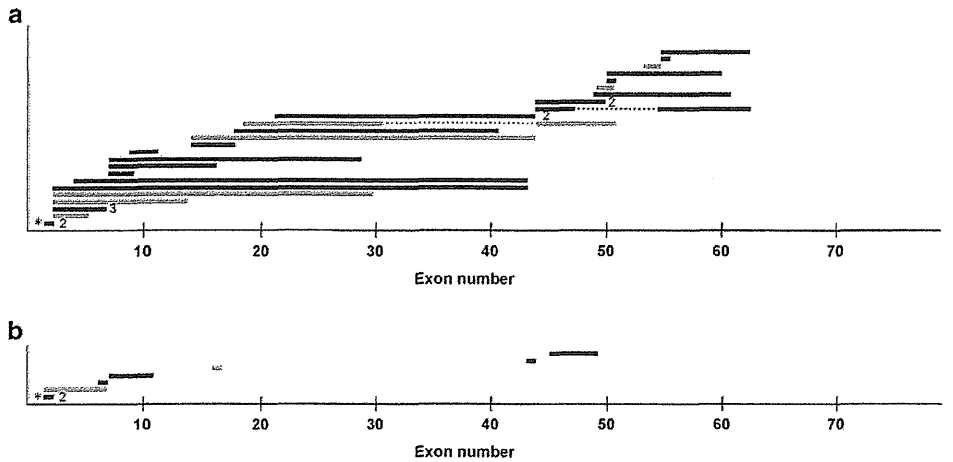

Figure 3 Patterns of exon duplication in DMD (**a**) and BMD cases (**b**). Exon duplications were categorized into 31 patterns from 38 cases. Bars represent the location of duplicated exons and filled and shaded bars represent out-of-frame or in-frame duplications, respectively. On the ninth and eleventh lines from the top in **a**, dotted lines indicate normal exons between separated duplications (duplication of exons 45–48 and 55–63, and exons 19–31 and 45–51, respectively). The number at the end of each line represents the number of cases with an identical duplication, whereas no number means that the duplication is unique. Asterisks indicate the duplication mutations that could result in both DMD and BMD phenotypes.

affected by these mutations differed from mutation to mutation.[33] Exon skipping was occurring in 12 cases. Cryptic splice site activation occurred in 12 cases, and in 3 cases both exon skipping and cryptic splice site activation occurred.

Other mutations

Thirty four small mutations, ranging from 1 to 606 bp, were identified (Table 3) and all were unique. Their locations were dispersed and no clustering was identified. Deletions ranging from 1 to 52 bp were identified in 25 cases. Insertions ranging from 1 to 606 bp were identified in nine cases. Two insertions, which were 606 bp within exon 44 and 325 bp within exon 67, respectively, were found to be identical in DNA sequence to retrotransposons.[34]

In one BMD case a splicing error was detected, although there was no mutation in the gDNA (KUCG no. 338 in Table 4).[26] Two previously unreported chromosomal abnormalities were found, once each (Table 4).

The frequency of small mutation identification was examined in all exons and their neighboring introns. Small mutations were identified six times in exon 44, and five times each in exons 8, 34 and 70. The length of exons 8, 34, 44 and 70 was 182, 171, 148 and 137 bp, respectively; that is, both longer and shorter than the mean dystrophin exon length of 143 bp, and hence the high mutation frequency in these exons does not reflect exon length. This suggests an unknown mutagenic susceptibility within these exons.

Genotype–phenotype correlations

The reading frame rule was examined in these deletion cases (Figure 2). Out of 270 cases, 252 complied with the reading frame rule (93.3%), whereas 18 cases (6.7%) did not: 9 DMD cases possessed an in-frame deletion (Figure 2a) and 9 BMD cases showed an out-of-frame deletion (Figure 2b). The exception to the rule was observed more frequently in BMD (15.5%) than in DMD (4.2%). It is remarkable that identical deletions could result in two different phenotypes; 3 out of 9 cases with exons 3–7 deletions, 2 out of 3 cases with exon 43 deletions, 1 out of 10 cases with exon 44 deletions and 2 out of 10 cases with exon 45 deletions show mild phenotype in spite of out-of-frame deletion, and 1 out of 2 cases with exons 10–44 deletions show severe phenotype in spite of in-frame deletion.

Among duplication mutations, 25 complied with the reading frame rule (65.8%) but 13 did not (34.2%): 7 DMD cases showed an in-frame duplication (Figure 3a) and 6 BMD cases had an out-of-frame duplication (Figure 3b). The incidence of cases that did not fit with the reading frame rule was higher among duplications (34.2%) than among the deletion mutations (6.7%). Dual phenotypes with an identical duplication were observed for exon 2 duplication.

DISCUSSION

In this study, pathogenic mutations in the dystrophin gene were successfully identified in all 442 Japanese DMD/BMD cases (Figure 1), and 49 (39%) of the identified small mutations were novel (Tables 1–3). This has enabled all Japanese dystrophinopathy cases to receive a proper diagnosis or genetic counseling and will also expedite the application of mutation-specific molecular therapies. The KUCG database is one of the largest mutation databases constructed at a single institute and is characterized by its high quality, because all the dystrophin mutations were identified and confirmed within one institute under a single principal investigator, and the clinical data from all patients are regularly updated at follow-up visits to the outpatient clinic.

Several mutation studies analyzing more than 100 DMD/BMD cases have been reported in the literature. The largest institutional database reported was from Italy; that database contained 506 cases, but some cases remained for which no dystrophin mutation had been identified.[35] The KUCG database is the second-largest institutional database, but the first completed mutation database. Even in small-scale studies, the mutation detection rate has previously only reached up to 96% of examined cases.[10,11,36,37] It was believed that a causative mutation in the dystrophin gene could not be identified in approximately 1 to 2% of DMD/BMD patients.[12] In contrast to previous studies, our perfect mutation detection rate became possible by conducting dystrophin cDNA or chromosomal analysis. Without these analyses, our mutation detection rate decreased by nearly 2%, that is, down to the previously accepted detection rate.[12] We propose

Table 1 Nonsense mutations detected in dystrophinopathy cases

KUCG no.	*Disease*	*Exon*	*DNA change*	*Protein change*
58	DMD	5	c.354G>A	p.Trp118X
571	DMD	5	c.355C>T	p.Gln119X
412	DMD	6	c.433C>T	p.Arg145X
5	DMD	7	c.580C>T	p.Gln194X
272	DMD	8	c.686T>A	p.Leu229X
65	DMD	8	c.724C>T	p.Gln242X
306	DMD	8	c.754G>T	p.Glu252X
377	DMD	10	c.1062G>A	p.Trp354X
171	DMD	10	c.1087C>T	p.Gln363X
9642	DMD	11	c.1324C>T	p.Gln442X
6	DMD	12	c.1474C>T	p.Gln492X
454	DMD	14	*c.1684C>T	p.Gln562X
496	DMD	15	c.1793C>G	p.Ser598X
582	DMD	17	*c.2047G>T	p.Glu683X
264	DMD	17	*c.2089A>T	p.Lys697X
102	DMD	18	c.2236G>T	p.Glu746X
78	DMD	19	c.2302C>T	p.Arg768X
85	DMD	19	c.2365G>T	p.Glu789X
482	DMD	19	c.2365G>T	p.Glu789X
847	DMD	20	c.2419C>T	p. Gln807X
203	DMD	20	c.2449C>T	p.Gln817X
809	DMD	21	*c.2677C>T	p.Gln893X
9695	DMD	21	c.2776C>T	p.Gln926X
326	DMD	23	c.3151C>T	p.Arg1051X
9625	DMD	26	*c.3472A>T	p.Lys1158X
54	DMD	26	c.3562A>T	p.Lys1188X
28	DMD	26	c.3580C>T	p.Gln1194X
9565	BMD	27	c.3631G>T	p.[Glu1211X, Arg1202_Glu1262del]**
522	DMD	28	*c.3795G>A	p.Trp1265X
797	BMD	31	c.4303G>T	p.[Glu1435X, Lys1412_Gln1448del]**
509	DMD	32	c.4375C>T	p.Arg1459X
594	DMD	32	c.4384C>T	p.Gln1462X
877	DMD	32	c.4414C>T	p.Gln1472X
652	DMD	33	*c.4527T>G	p.Tyr1509X
517	DMD	33	*c.4666G>T	p.Gly1556X
754	DMD	34	*c.4687A>T	p.Lys1563X
44	DMD	34	c.4729C>T	p.Arg1577X
539	DMD	34	c.4729C>T	p.Arg1577X
52	DMD	34	c.4757G>A	p.Trp1586X
4	DMD	36	c.5044G>T	p.Glu1682X
148	DMD	38	c.5350G>T	p.Glu1784X
577	DMD	39	c.5551C>T	p.Gln1851X
225	DMD	41	c.5899C>T	p.Arg1967X
501	DMD	41	c.5899C>T	p.Arg1967X
651	DMD	41	c.5899C>T	p.Arg1967X
593	DMD	42	c.5985T>G	p.Tyr1995X
30	DMD	43	c.6283C>T	p.Arg2095X
147	DMD	44	c.6423C>A	p.Tyr2141X
185	DMD	44	*c.6432T>A	p.Tyr2144X
341	DMD	47	c.6805C>T	p.Gln2269X
9584	DMD	47	*c.6862C>T	p.Gln2288X
700	DMD	50	*c.7255G>T	p.Glu2419X
456	DMD	53	*c.7817G>A	p.Trp2606X
475	DMD	53	*c.7855A>T	p.Lys2619X
314	DMD	57	c.8420G>A	p.Trp2807X
643	DMD	57	c.8460G>A	p.Trp2820X
550	DMD	58	c.8608C>T	p.Arg2870X
9627	DMD	58	c.8608C>T	p.Arg2870X
9655	DMD	59	*c.8746G>T	p.Glu2916X
720	DMD	62	*c.9216C>G	p.Tyr3072X
514	DMD	66	c.9568C>T	p.Arg3190X
724	DMD	66	c.9568C>T	p.Arg3190X
445	DMD	68	*c.9851G>A	p.Trp3284X
478	DMD	68	*c.9913G>T	p.Glu3305X
703	DMD	70	c.10108C>T	p.Arg3370X
852	DMD	70	c.10108C>T	p.Arg3370X
888	DMD	70	c.10141 C>T	p.Arg3381X
401	DMD	70	c.10171C>T	p.Arg3391X
9622	BMD	76	c.10873C>T	p.Gln3625X

Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; KUCG, Kobe University Clinical Genetics.
Asterisks indicate novel mutations that have not been previously reported in the Leiden Muscular Dystrophy database (updated 23 December 2009) (http://www.dmd.nl/), and double asterisks indicate nonsense mutations that resulted in splicing abnormalities (r.[3631g>u, 3604_3786del]) in KUCG no. 9565 and (r.[4303g>u, 4234_4344del]) in KUCG no. 797.

that similarly extensive mutation analysis should be performed to identify the responsible mutation when dystrophin deficiency has been confirmed.

An existing locus-specific database for dystrophinopathy in Leiden focuses on mutations that have been either reported in the literature or directly submitted to the database.[38] Because of the involvement of multiple submitters, the data quality is not homogeneous and analyses could be biased. The French database (UMD-DMD France), containing 2084 independent mutations in dystrophin, has been established by collaboration with several institutes, and the quality of UMD-DMD France is guaranteed by curators.[29]

For a long time, multiplex PCR examining selected deletion-prone exons has been used as the mutation screening method of choice for the dystrophin gene. Our results indicate that using MLPA to examine all 79 exons for deletion or duplication mutations allows mutation detection in 70% of Japanese DMD/BMD cases (Figure 1). In addition, MLPA has been shown to detect small mutations.[20] MLPA is considered to be the most powerful single technology to identify mutations in the huge dystrophin gene, and should be used as the first-line mutation detection method for the dystrophin gene in Japanese dystrophinopathy. In contrast, in Taiwan, which is geographically near to Japan, MLPA analysis identified a lower incidence of deletions and a higher incidence of duplications (deletion and duplication mutations were found in 36.0 and 24.7% of 89 DMD/BMD patients, respectively[39]), compared with other reports that show 60% and 5–10%, respectively.[6,8,40] Although differences in ethnicity should be taken into account, MLPA remains the first line in mutation detection in dystrophinopathy.[6–9]

A total of four deep intron mutations were identified at least 285 bp from the exon/intron boundary; this was possible only because we used detection of novel dystrophin mRNA (Figure 4). This analysis provided 1% of detected mutations, and facilitated our perfect mutation detection rate. Therefore, we suggest that it is necessary to analyze dystrophin mRNA to complete the mutation analysis, even when the dystrophin gene is apparently normal. Deep intron mutations have previously been identified in three cases, and their potential to be treated by exon skipping strategies was examined. Because the completely normal 79 exons of the dystrophin gene were maintained in these cases, induction of pseudoexon skipping would be expected to result in completely normal expression of dystrophin, the ultimate goal of dystrophinopathy treatment.[41] Our cases can now be tested for any potential to be treated by exon skipping.

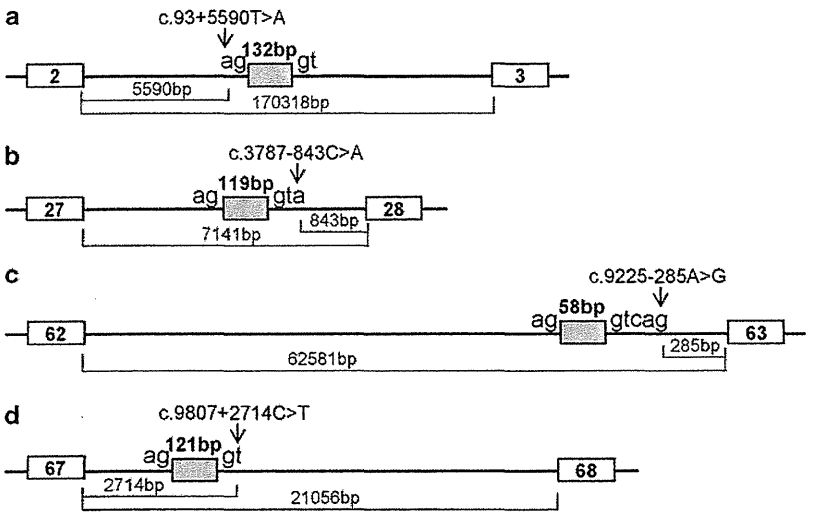

Figure 4 Deep intron mutations. Four deep intron mutations are schematically described. Boxes and lines represent exons and introns, respectively. The shaded boxes represent pseudoexons. Numbers in the open boxes indicate the exon number of the dystrophin gene, and the size of the pseudoexon is indicated above the shaded box. Two to five nucleotides adjacent to the pseudoexons are shown on the intron lines, and the mutated nucleotides are shown by vertical arrows. The upper and lower horizontal square brackets indicate distance from the intron/exon border and intron size, respectively. (**a**) In a BMD case (KUCG no. 9597), a novel splice site was created and a 132-bp exon was created and incorporated into the mRNA. (**b**) In a DMD case (KUCG no. 465), a novel splice site was created and a 119-bp exon was created and incorporated into the mRNA. (**c**) In a BMD case (KUCG no. 290), a novel splice site was created, and a 58-bp exon was created and incorporated into the mRNA. (**d**) In a DMD case (KUCG no. 857), a novel splice site was created, and a 121-bp exon was created and incorporated into the mRNA.

Table 2 Splice consensus site mutations in dystrophinopathy cases

KUCG no.	*Disease*	*Intron*	*DNA change*	*RNA change*
641	DMD	4	*c.264+2_264+3insGT	r.264_265insgu
712	DMD	11	*c.1329_1331+5delCAAGTAAG	r.[1150_1331del, 1150_1482del]
9602	BMD	13	c.1602G>T	r.1483_1602del
557	BMD	13	*c.1603-1G>T	r.[1603_1612del, 1603_1704del, 1603_1812del, 1603_1992del, 1603_2168del]
294	DMD	17	c.2168+1G>C	r.1993_2168del
9582	BMD	20	*c.2622G>C	r.[2622g>c, 2603_2622del]
682	DMD	20	c.2622+1G>A	r.2603_2622del
109	BMD	20	c.2623-2A>G	r.[2622_2623ins2623-7_2623-1; 2623-2a>g, 2622_2623ins2623-6_2623-1; 2623-2a>g, 2623_2629del]
687	DMD	21	*c.2804-2A>G	r.2803_2804insg
194	BMD	24	*c.3276+2T>G	r.[3276_3277ins3276+1-3276+39; 3276+2u>g, 3276_3277ins3276+1–3276+39; 3276+2u>g; 3277_3432del]
293	BMD	25	c.3432+1G>A	r.3277_3432del
739	BMD	29	c.4071+1G>C	r. 3787_4071del
329	DMD	32	c.4518+5G>A	r.[4518_4519ins4518+1_4518+28; 4518+5g>a, 4421_4518del]
154	BMD	42	*c.6117G>A	r.[5923_6117del, 6117_6118ins6117+1_6117+35; 6117g>a]
684	DMD	43	*c.6291-1G>A	r.6291_6438del
421	DMD	44	*c.6438+2T>A	r.spl?
701	DMD	45	*c.6614+1G>A	r.6583_6614del
391	DMD	45	*c.6615-2A>T	r.spl?
699	DMD	56	*c.8391-1G>A	r.[8391delg, 8391_8547del]
715	DMD	58	*c.8669-1G>C	r.8669_8670delag
220	DMD	65	c.9563+1G>A	r.spl?
9666	DMD	66	c.9649+2T>C	r.9564_9649del
9629	DMD	66	c.9649+5G>T	r.9564_9649del
775	DMD	70	c.10223+1G>A	r.10087_10223del

Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; KUCG, Kobe University Clinical Genetics.
Asterisks indicate the novel mutations that have not been previously reported in the Leiden Muscular Dystrophy database (updated 23 December 2009) (http://www.dmd.nl/).

In contrast to the deletions and duplications, which were localized within two hotspots, nonsense mutations were detected throughout the gene, with 30.4% (21), 30.4% (21), 24.6% (17) and 14.5% (10) in the first (exons 1–20), second (exons 21–40), third (exons 41–60) and fourth (exons 61–79) quartiles of the dystrophin gene, respectively. Although nonsense mutations were detected in 16% of all cases in

Table 3 Small insertion/deletion mutations from 1 to 606 nucleotides in dystrophinopathy cases

KUCG no.	*Disease*	*Exon*	*DNA change*	*Protein change*
212	BMD	2	c.53delA	p.Lys18ArgfsX8
39	DMD	5	c.280delA	p.Ile94LeufsX7
723	BMD	8	c.676_678delAAG	p.Lys226del
726	DMD	8	c.783dupT	p.Lys262X
499	DMD	12	*c.1373_1374delAA	p.Lys458ArgfsX4
231	DMD	12	*c.1376_1377delAG	p.Glu459ValfsX3
728	BMD	14	*c.1627delA	p.[Ile543SerfsX40, Val535_Gln664del]
277	DMD	15	*c.1773delA	p.Asp592IlefsX15
9600	DMD	19	c.2327_2378del52	p.Ala765ArgfsX15**
434	DMD	25	c.3347_3350delAGAA	p.Lys1116MetfsX36
579	BMD	27	*c.3613delG	p.[Glu1205LysfsX10, Arg1202_Glu1262del]**
492	DMD	27	*c.3766_3767insT	p.Gly1256ValfsX15
818	DMD	28	*c.3908_3909delCT	p.Ser1303X
245	DMD	29	*c.3959delC	p.Pro1320GlnfsX11
307	DMD	30	*c.4231dupC	p.Gln1411ProfsX6
795	DMD	33	c.4536-4540delGAGTG	p.Lys1516X
505	DMD	34	*c.4729delC	p.Arg1577GlufsX4
764	DMD	36	*c.5071dupA	p.Ile1691AsnfsX9
673	DMD	36	c.5140delG	p.Glu1714LysfsX7
414	BMD	38	c.5434_5437delTTCA	p.Ala1776_Met1816del**
436	DMD	39	*c.5561delT	p.Leu1854TyrfsX11
781	DMD	44	*c.6430dupT	p.Tyr2144LeufsX3
9601	DMD	44	c.6435_6436delins606	p.Arg2098AsnfxX16**
512	DMD	45	*c.6613dupA	p.Arg2205LysfsX18
404	DMD	49	*c.7128delA	p.Gln2376HisfsX24
638	DMD	50	*c.7221_7227delCTCTGAG	p.Ser2408GlyfsX4
342	DMD	52	*c.7654delG	p.Asp2552IlefsX24
836	DMD	59	*c.8733delC	p.Asn2912IlefsX7
761	DMD	63	c.9262delA	p.Met3088X
732	DMD	67	c.9657_9658ins325	p.Tyr3217CysfsX2**
289	DMD	71	c.10224dupT	p.Pro3409SerfsX24
702	BMD	74	c.10453delC	p.Leu3485X
441	DMD	74	c.10498_10499delAG	p.Ser3500X
9696	DMD	74	c.10498_10499delAG	p.Ser3500X

Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; KUCG, Kobe University Clinical Genetics.
Asterisks indicate the novel mutations that have not been previously reported in the Leiden Muscular Dystrophy database (updated 23 December 2009) (http://www.dmd.nl/), and double asterisks indicate small deletion/insertion mutations that resulted in splicing abnormalities (KUCG no. 9600: r.2293_2380del, KUCG no. 579: r.[3613delg, 3604_3786del], KUCG no. 414: r.5326_5448del, KUCG no. 9601: r.6291_6438del and KUCG no. 732: r.9650_9807del).

Table 4 Cases with chromosomal abnormalities and splicing errors without any mutation in genomic DNA

KUCG no.	*Disease*	*Mutation*
233	DMD	del(X)(p21.2-3)
481	DMD	inv(X)(p21.2q28)
338	BMD	c.=(r.10224_10553del)

Abbreviations: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; KUCG, Kobe University Clinical Genetics.

Japan, reports from outside Japan have described lower nonsense mutation rates up to 13.2%.[10,29,42] The rate of nonsense mutation is high among the Japanese. This may be because of a difference in the effort expended in finding nonsense mutations in these reports.

Table 5 Applicable cases for molecular therapies

Molecular therapies	*Applicable cases*
Nonsense suppression	66 (19%)
Skipping of	
Exon 51	40 (11%)
Exon 53	39 (11%)
Exon 45	31 (9%)
Exon 44	14 (4%)
Exon 8	12 (3%)
Exon 43	10 (3%)
Exon 52	10 (3%)
Exon 55	10 (3%)

It has been reported that one nonsense mutation at a CpG site in exon 59 (c.8713C>T; p.R2905X) was detected in six patients with different haplotypes,[28] and this mutation has been reported from several countries, suggesting that c.8713C>T is a hotspot for mutation. In this study, however, no case showed this point mutation. In contrast, in the Japanese, the most common nucleotide change was c.5899C>T, which was found in three cases (Table 1). Interestingly, c.5899C>T has been once detected in the United States.[28] In Taiwan, which is geographically near to Japan, c.10108C>T was identified three times among 14 nonsense mutation cases,[42] and this mutation was identified twice in our study. These findings suggest an ethnic difference in the mutability of CpG sites, involving the rate of demethylation or features of the chromatin structure.

Although the enormous dystrophin gene has huge introns that have accumulated retrotransposon insertions, this has rarely been shown to cause disease.[43] In our series, two retrotransposon insertions were found.[34] Their identification was because of the elongation of exon size, which made PCR amplification difficult, and could have resulted in misdiagnosis as a single-exon deletion. Therefore, we propose that supposed single-exon deletion cases should be examined in more detail.

Although the reading frame rule is thought to explain the phenotype,[5] 18 out of 270 cases with deletion (6.7%) and 13 out of 38 cases with duplication (34.2%) did not fit with the reading frame rule (Figures 2 and 3). Furthermore, identical mutations could result in two different phenotypes; deletions of exons 3–7, 10-44, 43, 44 and 45, and duplication of exon 2. In-frame deletions starting in the actin-binding domain or disrupting the cysteine-rich domain resulted in DMD phenotype because of the lack of binding sites with dystrophin-associated proteins. Other mechanisms may have a role in modulating the clinical severity, including recoding mechanism and unusual splicing.[29]

We examined the applicability for molecular therapies among the 260 different mutations identified in DMD/BMD (Table 5). Nonsense suppression therapy has been studied and a compound named PTC124 is now in clinical trials to treat DMD cases caused by a nonsense mutation.[15] This treatment could be applied to 66 cases with nonsense mutations in our study (19% of DMD cases). Induction of exon skipping that corrects out-of-frame to in-frame has been proposed as a highly plausible method for DMD treatment, and the first clinical trial has been conducted in Japan.[13,44] The applicability of this technology was examined in the KUCG database. The greatest number of treatable cases with one antisense oligonucleotide was 40, which could be treated by skipping of the single exon 51. In fact, exon

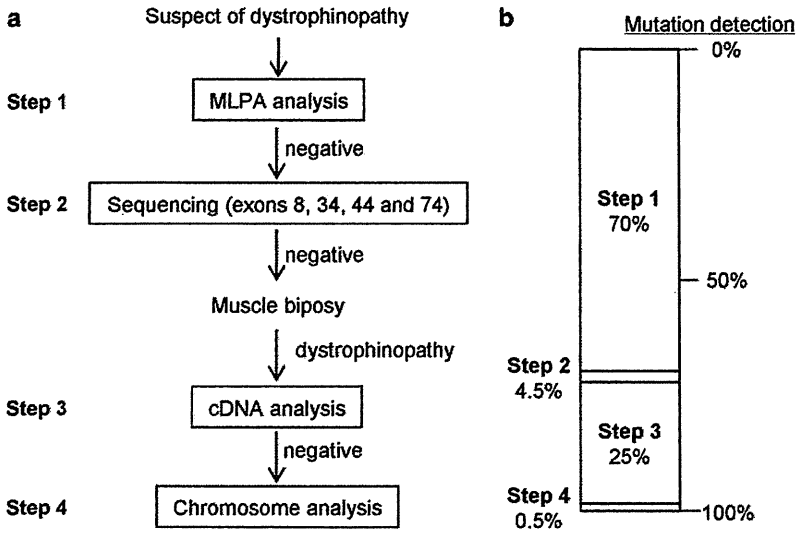

Figure 5 Molecular testing strategy for Japanese dystrophinopathy. A diagram for the molecular diagnosis of dystrophinopathy (a) and the frequency of mutation detection by each diagnostic step (b). Multiplex ligation-dependent probe amplification (MLPA) analysis identifies deletion and duplication mutations encompassing one or more exons, which account for 70% of dystrophinopathy cases. In cases with loss of a single exon by MLPA, the exon structure was examined by another method before concluding that there is a single-exon deletion, because small mutations have been found to disturb MLPA amplification.[20] Sequencing of exons and flanking introns of exons 8, 34, 44 and 74, in which small mutations are detected frequently, revealed mutations in a further 4.5% of dystrophinopathy cases. After confirmation of the diagnosis as dystrophinopathy, cDNA analysis was performed, which identified mutations in 25% of cases. After chromosome analysis, mutations were identified in all dystrophinopathy cases.

51 skipping treatment has already been clinically tested in Europe.[14] Skipping of exon 53 would produce an in-frame transcript in 39 DMD cases. In total, single-exon skipping could be applied to the treatment of 173 cases with 58 patterns of deletion. Skipping of two or three exons could be applied to 27 and 1 case, respectively. It seems therefore that induction of exon skipping is the most applicable molecular therapy for the treatment of DMD. However, it is noteworthy that dual phenotypes can result from one identical deletion (Figures 2a and b), and this must be considered when assessing the potential effects of the treatment. With exon skipping treatment, the most effective results would be expected for the four deep intron mutations (Figure 4), because expression of a completely normal dystrophin transcript could be induced.

The best strategy for comprehensive dystrophin gene testing has long been debated. A balance must be found between the need to obtain definitive results and the effort required to perform a comprehensive analysis of heterogeneous mutations in the large dystrophin gene.[10] Recently, one analytical method has been shown to identify mutations in the dystrophin gene in 98% of DMD cases.[37] This is very useful but is still not perfect in identifying all mutations. From our results, it is possible to define a strategy that will be useful in prioritizing cases for various stages of dystrophin gene mutation analysis and will maximize the benefits of comprehensive mutation analysis for dystrophinopathy (Figure 5). In this strategy, MLPA analysis should be used first, to detect 70% of the mutations (step 1). Second, direct sequencing of the four exons 8, 34, 44 and 70 (which each contained small mutations in five or six cases; Tables 1–3) should be conducted to reveal mutations in a further 4.5% of cases (step 2). Thereafter, muscle biopsy should be conducted to confirm the diagnosis and obtain dystrophin mRNA for sequence analysis. Dystrophin cDNA analysis is expected to reveal mutations in 25% of cases (step 3). Finally, chromosome analysis would complete the mutation analysis (step 4), enabling mutation detection in all dystrophinopathy cases.

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RESEARCH ARTICLE Open Access

Low incidence of limb-girdle muscular dystrophy type 2C revealed by a mutation study in Japanese patients clinically diagnosed with DMD

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Abstract

Background: Limb-girdle muscular dystrophy type 2C (LGMD2C) is an autosomal recessive muscle dystrophy that resembles Duchenne muscular dystrophy (DMD). Although DMD is known to affect one in every 3500 males regardless of race, a widespread founder mutation causing LGMD2C has been described in North Africa. However, the incidence of LGMD2C in Japanese has been unknown because the genetic background remains uncharacterized in many patients clinically diagnosed with DMD.

Methods: We enrolled 324 patients referred to the Kobe University Hospital with suspected DMD. Mutations in the dystrophin or the SGCG genes were analyzed using not only genomic DNA but also cDNA.

Results: In 322 of the 324 patients, responsible mutations in the dystrophin were successfully revealed, confirming DMD diagnosis. The remaining two patients had normal dystrophin expression but absence of γ-sarcoglycan in skeletal muscle. Mutation analysis of the SGCG gene revealed homozygous deletion of exon 6 in one patient, while the other had a novel single nucleotide insertion in exon 7 in one allele and deletion of exon 6 in the other allele. These mutations created a stop codon that led to a γ-sarcoglycan deficiency, and we therefore diagnosed these two patients as having LGMD2C. Thus, the relative incidence of LGMD2C among Japanese DMD-like patients can be calculated as 1 in 161 patients suspected to have DMD (2 of 324 patients = 0.6%). Taking into consideration the DMD incidence for the overall population (1/3,500 males), the incidence of LGMD2C can be estimated as 1 per 560,000 or 1.8 per million.

Conclusions: To the best of our knowledge, this is the first study to demonstrate a low incidence of LGMD2C in the Japanese population.

Background

Duchenne muscular dystrophy (DMD; OMIM#310200) is the most common inherited muscular dystrophy, and affects 1 in every 3,500 males, regardless of race. DMD is caused by a mutation in the dystrophin gene on the short arm of the X chromosome and is characterized by the absence of dystrophin in skeletal muscle. Those affected by DMD develop muscle weakness by the age of 4 or 5, followed by progressive muscle wasting that ultimately leads to patients being wheelchair bound by the age of 12. In addition, calf hypertrophy and lumbar lordosis are also observed. DMD patients succumb to either cardiac or respiratory failure secondary to the disease during their twenties [1].

Limb-girdle muscular dystrophy type 2C (LGMD2C) (OMIM # 253700) is an autosomal recessive disorder caused by mutations in the SGCG gene, which encodes γ-sarcoglycan. It is characterized by a childhood onset of progressive muscular dystrophy. The mean age of onset is 5.3 years, and half of these patients lose ambulation by the age of 12. Calf hypertrophy and lumbar lordosis are common [2]. Based on these clinical findings, LGMD2C is referred to as a severe childhood autosomal recessive muscular dystrophy or as a Duchenne muscular dystrophy (DMD)-like autosomal recessive disease [3].

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Unlike DMD, LGMD2C shows geographical difference in its incidence. The highest incidence of LGMD2C has been reported in North Africa as a result of a founder mutation in the SGCG gene [4]. Numerous studies have summarized the clinical and pathological features of LGMDs outside of North Africa. These studies have reported at least 19 subtypes, with 7 exhibiting autosomal dominant (LGMD1A to E) and 12 exhibiting autosomal recessive (LGMD2A to J) patterns of inheritance [5]. Previous studies have determined the prevalence of LGMD to range from 8.1 per million in a nationwide study in The Netherlands [6] to 40 per million in a worldwide survey [7]. However, the incidence of subtype LGMD2C has yet to be determined. In the Bulgarian Roma (Gypsy) population, one founder mutation has been reported to be common [8]. Other than in the geographical areas associated with founder mutations, only limited numbers of LGMD2C cases have been reported. For example, only nine and seven LGMD2C patients have been described among large numbers of patients examined in Italy [5] and the USA [9], respectively.

Since differentiation of LGMD2C from DMD has not been considered a major problem in current clinical practice, strong efforts to differentiate the two conditions have not been made. Dystrophin restoration therapy for DMD by either inducing exon skipping [10,11] or by suppressing nonsense mutations [12] appears to be close to clinical implementation. However, before there can be any clinical application of these technologies, it is essential that DMD be confirmed at the molecular level.

Kobe University Hospital contains a DMD clinic that examines patients suspected to have the disease from all over Japan, especially from the western part of the country. We herein report on two LDMD2C patients that were found among a group of 324 Japanese patients suspected to have DMD. We accordingly estimate the incidence of LGMD2C in the Japanese population to be 1 per 560,000.

Methods

Patients

Boys were enrolled in this cohort if they had been referred to the Kobe University Hospital with a tentative clinical DMD diagnosis based on strongly elevated levels of serum creatine kinase (CK) activity. Patients ranged in age from 0 - 7 years. We performed an extensive analysis on mutations in the dystrophin gene and were able to molecularly identify a mutation in the dystrophin gene in 322 patients with DMD-like disease (manuscript in preparation). However, in two other patients, no mutations were noted in the dystrophin gene. After obtaining informed consent from their parents, further examinations were conducted on these patients.

Methods

Muscle biopsy

Muscle samples were obtained from the quadriceps of each patient. Standard histochemical stains including hematoxylin and eosin (H-E), Gomori trichrome, NADH tetrazolium reductase, succinate dehydrogenase, periodic acid-Schiff, acid phosphatase, adenosine triphosphatase at pH 4.3 and 9.4, cytochrome c oxidase, and alkaline phosphatase were conducted. Immunohistochemical stains for α- and β-dystroglycan; α-, β-, γ-, and δ-sarcoglycan; dystrophin; and merosin were performed using their respective monoclonal antibodies (Novocastra, Newcastle upon Tyne, United Kingdom, and Millipore, Billerica, MA, USA).

Gene analysis

Under an institutionally approved protocol, DNA was extracted from blood samples that were obtained from the probands and all available family members. For examination of the SGCG gene, eight sets of primers (Table 1) were designed to amplify eight exons, with the amplified PCR products then directly sequenced. Reverse transcription PCR (RT-PCR) was used to analyze the SGCG mRNA expressed in lymphocytes or skeletal muscle, as previously described [13]. Full-length SGCG cDNA was amplified as two separate, partially overlapping fragments by using two sets of primers (Table 1). The amplified products were then directly sequenced.

Quantitative PCR

Genomic dosage of the exons of the SGCG gene was assessed by a semiquantitative multiplex PCR, as previously described [14]. Eight fragments encompassing exons 1 to 8 of the SGCG gene and one fragment encompassing exon 2 of the α-dystroglycan gene were co-amplified using two PCR reactions that employed six sets of primers (Table 1). PCR products were separated by capillary electrophoresis (Agilent 2001 Bioanalyzer with DNA 1000 Lab Chips, Agilent Technologies, Palo Alto, CA, USA). The amount of PCR product derived from the SGCG exons was quantified by measuring their peak areas followed by calculating the ratio of these areas to that found for the α-dystroglycan exon 2.

Results

The two male patients with clinical diagnosis of DMD were incidentally found to have marked elevations of serum CK levels (more than 50 times higher than control) in early childhood, despite a negative family history for muscular dystrophy. To confirm the clinical diagnosis of DMD, dystrophin gene mutations were extensively searched for using not only genomic DNA but also mRNA. However, no mutations could be identified, even when we included a deep intron mutation [15].

To clarify pathological changes in these two patients, muscle biopsies were performed. In patient 1 (KUCG