was added to 15 μl of each reaction mixture and incubated at 37 °C for 1 h. After the mixture was filtrated with an Ultrafree-MC column (Millipore, Bedford, MA), a 15 μl aliquot was subjected to phase-high performance liquid chromatography (HPLC) on an ODS-80Ts QA column (4.6 × 250 mm; Tosoh, Tokyo, Japan) and a GlycoSep N amide-adsorption column (GlycoSystems, Oxford) for DNS labeled substrate and 2AB labeled substrate, respectively. Acetonitrile (30%)/H_2O (70%) and 35% ammonium formate (pH 4.4)/65% acetonitrile were used as running solution at a flow rate of 1.0 ml/min at 30 °C for the analyses of DNS labeled substrate and 2AB labeled substrate, respectively. SCL-10A_{vp} (Shimazu, Kyoto, Japan) was used for detection of the peaks.

Assay of GalT activity toward GlcNAcβ1-2mannosylpeptide. The GalT activity was based on the amount of [^{3}H]Gal transferred to a GlcNAcβ1-2mannosylpeptide, Ac-Ala-Ala-Pro-(GlcNAcβ1-2Man) Thr-Pro-Val-Ala-Ala-Pro-NH_2. The acceptor substrate was prepared by the enzymatic reaction, GlcNAc transferred to a mannosylpeptide, Ac-Ala-Ala-Pro-(Man)Thr-Pro-Val-Ala-Ala-Pro-NH_2, using human protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) as described previously [7]. Briefly, a reaction mixture contained 140 mM Mes buffer (pH 7.0), 400 mM UDP-GlcNAc, 400 mM mannosylpeptide, 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol, and partially purified recombinant human POMGnT1 in 50 μl total volume. After being incubated for 72 h at 37 °C, the GlcNAcβ1-2mannosylpeptide was separated by a Wakopak 5C18-200 column (4.6 × 250 mm; Wako Pure Chemical Ind., Osaka). Solvent A was 0.1% trifluoroacetic acid in distilled water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–25% solvent B and monitored continuously at 215 nm.

The GalT assay was performed in a mixture of 10 mM Hepes buffer (pH 7.4), 0.2 mM UDP-[^{3}H]Gal (240,000 dpm/nmol; New England Nuclear, Boston, MA), 0.4 mM acceptor substrate (GlcNAcβ1-2mannosylpeptide), 10 mM $MnCl_2$, and purified enzyme solution in 50 μl total volume. After being incubated for 2 h at 37 °C, the product was separated by HPLC as described above and the radioactivity of each fraction was measured using a liquid scintillation counter.

Quantitative analysis of human β4GalTs transcript in human normal tissues by real time PCR. Total RNA was extracted from human tissues by the methods of Chomczynski and Sacchi. First-strand cDNA was synthesized using a SuperscriptII first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions. Quantitation of each human *β4GalTs* transcript expression was performed by real time PCR using the following primers and probe: *T-I*, forward 5′-TCACAAGGTGGCCATCATCA-3′, reverse 5′-GCAGGACTGGGTGCAAATAATAT-3′, and probe 5′-TCCATTCCGCAACCGGCAGG-3′; *T-II*, forward 5′-CAACCAGCATGGTGAGGACA-3′, reverse 5′-AGCGCCTCTAGGAAGCCC-3′, and probe 5′-CAACCGGGCCAAGCTGCTTAACG-3′; *T-III*, forward 5′-CCATGTTGCCGTTGCTATGA-3′, reverse 5′-AGTGCTGAGACTCCTCCGAAGT-3′, and probe 5′-TGGATACAGCCTCCCGTACCCCCA-3′; *T-IV*, forward 5′-ACTTGACAACTGCCCTTCTGTGT-3′, reverse 5′-CAAAGTGAGATCTGGTTTGAAAA-3′, and probe 5′-TCCTTACTCAGAGGCCAGAGCAAGCT-3′; *T-V*, forward 5′-CGCTGCTGTACTTCGTCTATGTG-3′, reverse 5′-CCTTGGGCTTGCATCATGA-3′, and probe 5′-CGCCCGGCATAGTGAACACCTACCT-3′; *T-VI*, forward 5′-CAGAAGCAGCGGCTGGAA-3′, reverse 5′-TCGCACGGTTAAAAGGTTGTG-3′, and probe 5′-GCCAGTCTGTTCAATGACATAAAACGCAA-3′; *T-VII*, forward 5′-CCACATCTACGTGCTCAACCA-3′, reverse 5′-TCTCCAGGAAGCCCACGTT-3′, and probe 5′-TGGACCACTTCAGGTTCAACCGGG-3′. The probe was labeled at the 5′-end with the reporter dye 3FAM, and at the 3′-end with the quencher dye TAMRA (Applied Biosystems, Foste City, CA). Real time PCR was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems). The relative amount of each human *β4GalTs* transcript was normalized by *GAPDH* transcript in the same cDNA.

Mutation analysis of β4GalT-I and β4GalT-II in the patients with CMD. We analyzed genomic DNA from 50 patients with CMD, brain malformation, and ocular abnormalities who have no *fukutin* or *POMGnT1* mutation. Primers to amplify each exon and surrounding intronic sequences were designed from the genomic sequence of the *β4GalT-I* and *β4GalT-II* genes. PCR products from patient genomic DNA were sequenced using Bigdye terminators (Applied Biosystems). Fragments were electrophoresed on an ABI Prism 3100 sequencer (Applied Biosystems).

Results and discussion

Acceptor substrate specificity of human β4GalTs

To facilitate the enzymatic analysis of glycosyltransferases, a soluble form of the protein was generated with immunoglobulin κ signal sequence and a FLAG tag, as described under Materials and methods. The soluble glycosyltransferase was expressed in *Sf-21* cells as a recombinant enzyme fused with the FLAG tag. The fused enzyme expressed in the cell culture supernatants was purified by anti-FLAG M1 antibody-conjugated resin and quantitated by Western blotting analysis using anti-FLAG antibody.

We determined the acceptor substrate specificity of the purified human β4GalTs by utilizing three oligosaccharide acceptor substrates, such as a disaccharide (GlcNAcβ1-2Man), a disaccharide (GlcNAcβ1-3Fuc), and poly-*N*-acetyllactosamine, and each product was analyzed on HPLC. The activities of the human β4GalTs toward three acceptor substrates are summarized in Table 1. Among human β4GalTs, β4GalT-II transferred Gal most efficiently to all substrates, and β4GalT-I was the second most efficient. The activity of β4GalT-II was about three times higher than that of β4GalT-I. Then, we examined the GalT activity toward a glycopeptide (GlcNAcβ1-2Man-peptide), to investigate the effect of peptide on specificity of the GalT activity. As a result in Fig. 1, β4GalT-II also transferred Gal most efficiently as mentioned above, although the relative activity of β4GalT-I toward β4GalT-II was higher than the above three substrates, suggesting that the peptide has a role for the specificity of β4GalTs.

β4GalT-II transferred Gal most efficiently toward both *O*-mannosyl glycans and *O*-mannosyl peptide, and β4GalT-I transferred the second most efficiently (Table 1 and Fig. 1). A sialyl *O*-mannosyl glycan, Siaα2-3Galβ1-4GlcNAcβ1-2Man, on α-DG is essential for a laminin-binding ligand, and several CMDs have been reported as being defective in the enzyme required for the synthesis of such *O*-mannosyl glycan [6–8]. Other candidate regulators of *O*-mannosyl glycan, such as Fukutin or Large, have been involved in CMD, but each glycosyltransferase activity has not yet been determined [30,31]. It has been reported that deficiency of *β4GalT-I* causes the congenital disorder of glycosylation (CDG)

Table 1
Acceptor substrate specificity of β4GalTs

Acceptor substrate	nmol/h/mg protein						
	T-I	T-II	T-III	T-IV	T-V	T-VI	T-VII
GlcNAcβ1-2Man-2AB	7.2[a]	25.3	2.5	0.4	0.1	0.6	ND[b]
GlcNAcβ1-3Fuc-DNS	101.0	286.0	8.8	2.0	2.6	17.9	ND
GlcNAcβ1-3(Galβ1-4GlcNAcβ1-3)$_2$-2AB	69.1	173.7	16.9	1.6	0.7	3.2	ND

[a] The values represent averages of two independent experiments.
[b] ND, not detected.

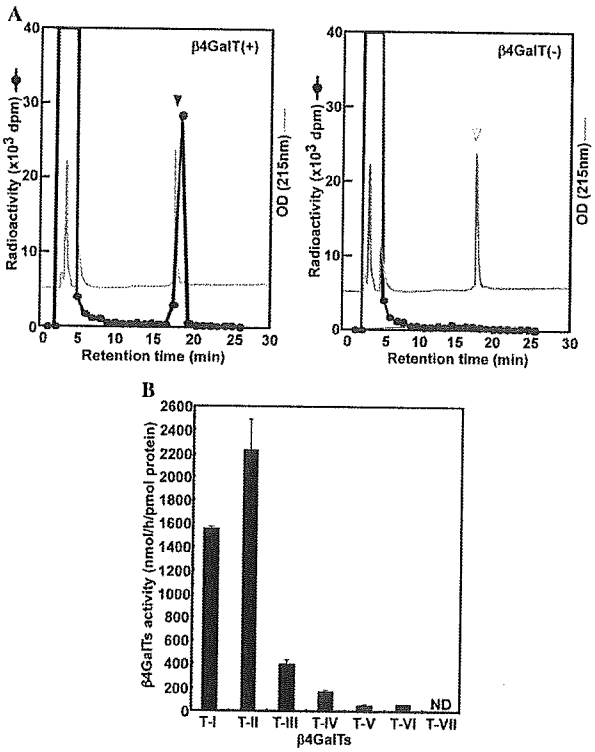

Fig. 1. Activities of human β4GalTs toward GlcNAcβ1-2mannosylpeptide. (A) Reversed phase-HPLC analyses of reaction products from GlcNAcβ1-2mannosylpeptide as an acceptor of UDP-[^{3}H]Gal with (left) or without (right) each human β4GalT. Eluting fractions were collected and their radioactivities were measured with a liquid scintillation counter. Typical example is shown here. The radioactivity and the absorbance of 215 nm are black line and gray line, respectively. The peak at 18.5 min (closed arrowhead and open arrowhead) corresponds to glycopeptide. The GalT activity was determined by incorporation of [^{3}H]Gal into GlcNAcβ1-2mannosylpeptide (closed arrowhead). (B) Activities of human β4GalTs toward GlcNAcβ1-2mannosylpeptide. Data represent the average of two independent experiments and are shown as mean values with standard errors.

type IId, with brain malformation, mental retardation, myopathy, and blood clotting defects [32], but the loss of sialic acid and galactose residues of *O*-mannosyl glycan has not been demonstrated. Also, CMD like phenotypes have not been reported in *β4GalT-I* knockout mice [33,34]. This suggests that β4GalT-II is a possible major regulator of the synthesis of *O*-mannosyl trisaccharide, Galβ1-4GlcNAcβ1-2Man, and may be involved in several CMDs.

Notch receptors are transmembrane glycoproteins, and these *O*-fucosyl glycans are essential for Notch signaling [11,12]. β4GalT acts on a disaccharide (GlcNAcβ1-3Fuc), and the other group demonstrated that among six β4GalTs, only β4GalT-I transferred Gal to the disaccharide in Chinese hamster ovary cells and modulated Notch signaling [35]. However, in our results, β4GalT-I and β4GalT-II transferred Gal efficiently in vitro, and the activity of β4GalT-II was about three times higher than that of β4GalT-I. These differences may be caused by differences of experimental sources such as in vivo or in vitro, and in vivo there is the possibility that the reduction of Notch signaling in *β4GalT-I* mutant cells may be caused by the deficiency of Gal addition to the other Notch signaling related molecules. In *β4GalT-I* knockout mice, growth retardation and semi-lethality before weaning are exhibited [33,34], but these phenotypes are not serious as compared with that of *Lunatic-fringe* deficiency [36], suggesting that another β4GalTs act on the disaccharide. Thus, β4GalT-II is another possible regulator of the elongation of the fringe disaccharide, GlcNAcβ1-3Fuc.

We also examined the GalT activity toward poly-*N*-acetyllactosamine in addition to the two substrates. As shown in Table 1, the activity of β4GalT-II toward poly-*N*-acetyllactosamine was about three times higher than that of β4GalT-I, although β4GalT-I also transferred Gal efficiently. Poly-*N*-acetyllactosamines are often modified to express differentiation antigens and functional oligosaccharides, such as Lewis^X (Le^X), polysialic acid (PSA), and human natural killer-1 (HNK-1) carbohydrate [37,38]. The Le^X Carbohydrate structure functions as a cell–cell recognition molecule in the highly organized structures of the central nervous system [39]. PSA and HNK-1 carbohydrate, both of which are expressed on the outer chain moieties of N-linked oligosaccharides of several neural cell adhesion proteins including the neural cell adhesion molecule [40,41], are involved in neuronal development [42,43]. It was suggested that β4GalT-II and/or β4GalT-V act on PSA and HNK-1 carbohydrate in mouse brain development

[44]. *β4GalT-I* knockout mice demonstrated that PSA and HNK-1 are expressed normally, and no neuronal defects are detected, although reduction of the synthesis of selectin-ligand, such as sialyl Lewis^X, resulted in the reduction of inflammatory responses [45]. Thus, β4GalT-II is a possible major regulator of the synthesis of poly-*N*-acetyllactosamine involved in neuronal development.

As mentioned above, β4GalT-II acted most efficiently on several oligosaccharides involved in the development of the nervous system among human β4GalTs. However, all β4GalTs except for β4GalT-VII show GalT activity toward each substrate, suggesting that several β4GalTs could function coordinately.

Quantitative analysis of human β4GalTs transcript in human normal tissues by real time PCR

We determined the tissue distribution and expression levels of human *β4GalTs* transcript by the real time PCR method, which is a sensitive and accurate assay system. The expression levels of human *β4GalTs* in various tissues were shown as relative values to the *GAPDH* transcript to be able to compare to each other (Fig. 2). Both *β4GalT-II* and *β4GalT-III* were expressed at high levels in brain, and especially in fetal brain (Fig. 2A), suggesting that those genes play important roles in neuronal development. In other reports [16,20], *β4GalT-II* was expressed weakly in adult brain. The discrepancy of *β4GalT-II* expression level in adult brain may be derived from the differences in cDNA sources and the analytical method. The activity of β4GalT-III to the substrates related to neuronal development in this report was much lower than that of β4GalT-II, and β4GalT-III was widely expressed at high levels in all human tissues (Fig. 2B). This suggests that β4GalT-III acts on, not glycans mentioned in this report, but glycolipids that existed in all cells. Moreover, *β4GalT-II* was expressed at highest levels in fetal brain, and this result corresponds with the suggestion of the importance of β4GalT-II in mouse brain development [44].

On the other hand, other *β4GalTs* involving *β4GalT-I* were expressed at very low levels in brain (Fig. 2A) compared with *β4GalT-II* (Fig. 2A), although both *β4GalT-I* and *β4GalT-II* expressed high activity toward substrates in this report as shown in Table 1 and Fig. 1. It was also reported that the levels of *β4GalT-I* did not correlate with the GalT activity levels in mouse brain [46]. These results suggest that β4GalT-II is a major regulator of the synthesis of glycans involved in the brain.

In other normal tissues, *β4GalTs* were expressed differentially at various levels (Fig. 2B). The distribution of each transcript corresponds with other reports [20,22], although there may be individual differences in cDNA sources. High steady state levels of *β4GalT-II* were seen not only in brain, but also in skeletal muscle

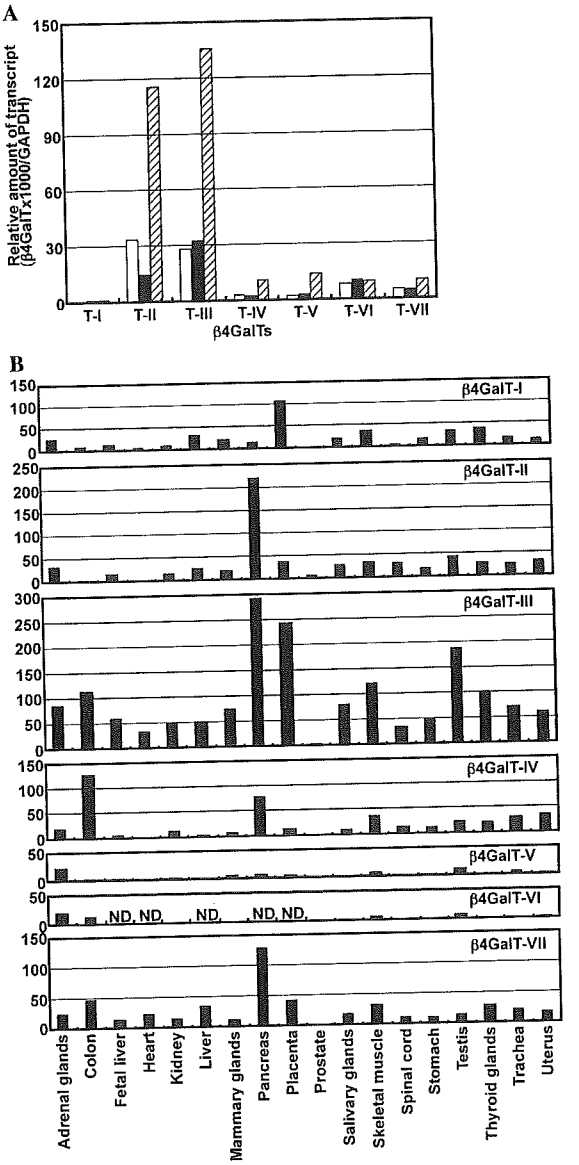

Fig. 2. Quantitative analysis of human *β4GalTs* transcript in human tissues by real time PCR. (A) The expression levels in brain (open bars, whole brain; solid bars, cerebellum; hatched bars, fetal brain). (B) The expression levels in 18 tissues. The expression levels of human *β4GalTs* transcripts were normalized to those of the *GAPDH* transcripts, which were measured in the same cDNAs, and their expression levels could be compared with each other. Two independent experiments were conducted and representative results are shown.

and testis. The distribution corresponds with that of protein *O-mannosyltransferase* 1 [47], which is involved in the synthesis of *O*-mannosyl glycan, and cause gene of Walker–Warburg syndrome characterized by CMD. So, transcript level and activity of *β4GalT-II* support the possibility that β4GalT-II acts on *O*-mannosyl glycan, and β4GalT-II may be involved in uncharacterized CMDs.

Analysis of β4GalT-I and β4GalT-II in the patients of CMDs

We examined the presence of mutations on the *β4GalT-I* and *β4GalT-II* genes of patients with CMDs whose causing gene was not identified, but we have detected no mutations in any of the 50 patients. As described above, CDG-IId has been observed in only one patient so far [32]. A possible explanation for the absence of *β4GalT-I* and *β4GalT-II* mutations in our subjects is that such patients may not be diagnosed as CMD because of their moderate symptoms. Another possibility is that patients with *β4GalT-I* and *β4GalT-II* mutations were simply not included in the 50 CMD patients studied here. A worldwide survey of the occurrence of *β4GalT-I* and *β4GalT-II* mutations is needed to determine whether any CMD is present to be caused by *β4GalT-I* and *β4GalT-II* mutations.

Conclusions

In the present study, we determined the acceptor specificity of human β4GalTs by utilizing substrates that are essential glycans for neuronal development, and the transcript expression levels in human normal tissues. Among human *β4GalTs*, *β4GalT-II* expressed the highest activity toward all four substrates. Moreover, the transcript levels of *β4GalT-II* were high in brain. These results suggest that β4GalT-II acts most efficiently of all human β4GalTs on glycans involved in neuronal development, such as a disaccharide (GlcNAcβ1-2Man) on α-DG and a disaccharide (GlcNAcβ1-3Fuc) on Notch receptors.

To determine further the function of β4GalT-II in biological events in neuronal development, *β4GalT-II* knockout mice should be generated in future. It may be necessary to knockout two or more *β4GalTs* in mice to elucidate the unknown function of the glycans involved in biological events as β4GalTs may function coordinately in the same tissues.

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Founder SVA Retrotransposal Insertion in Fukuyama-Type Congenital Muscular Dystrophy and Its Origin in Japanese and Northeast Asian Populations

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Fukuyama-type congenital muscular dystrophy (FCMD), one of the most common autosomal recessive disorders in Japan, is characterized by congenital muscular dystrophy associated with brain malformation due to a defect in neuronal migration. Previously, we identified the gene responsible for FCMD, which encodes the fukutin protein. Most FCMD-bearing chromosomes (87%) are derived from a single ancestral founder, who lived 2,000–2,500 years ago and whose mutation consisted of a 3-kb retrotransposal insertion in the 3′ non-coding region of the *fukutin* gene. Here we show, through detailed sequence analysis, that the founder insertion is derived from the SINE-VNTR-*Alu* (SVA) retroposon. To enable rapid detection of this insertion, we have developed a PCR-based diagnostic method that uses three primers simultaneously. We used this method to investigate the distribution and origin of the founder insertion, screening a total of 4,718 control DNA samples from Japanese and other Northeast Asian populations. Fifteen founder chromosomes were detected among 2,814 Japanese individuals. Heterozygous carriers were found in various regions throughout Japan, with an averaged ratio of 1 in 188. In Korean populations, we detected one carrier in 935 individuals. However, we were unable to detect any heterozygous alleles in 203 Mongolians and 766 Mainland Chinese populations. These data largely rule out the possibility that a single ancestor bearing an insertion-chromosome immigrated to Japan from Korea or Mainland China and appear to confirm that FCMD carriers are rare outside of Japan.

KEY WORDS: **Fukuyama-type congenital muscular dystrophy (FCMD); retrotransposon; SINE-VNTR-*Alu* (SVA); fukutin; Japanese; Northeast Asia; genetic epidemiology**

INTRODUCTION

In 1960, Fukuyama et al. first described a new type of congenital muscular dystrophy, named Fukuyama-type congenital muscular dystrophy (FCMD; MIM 253800) [Fukuyama et al., 1960]. An autosomal recessive disorder, FCMD is the second most common childhood muscular dystrophy in Japan, characterized by brain malformation (cortical dysgenesis, principally cerebral, and cerebellar cortical dysplasia), primary dystrophic changes in skeletal muscle, and mental retardation [Nonaka and Chou, 1979; Fukuyama et al., 1981; Fukuyama and Ohsawa, 1984]. This disease presents almost exclusively in Japanese infants. Most FCMD patients are incapable of standing or walking; a patient's peak motor function usually consists only of unassisted sitting or sliding on the buttocks. Intellectual, cognitive, and communicative functions are, without exception, moderately delayed, with the most common brain anomalies being micropolygyria, pachygyria, and agyria due to a defect in neuronal migration. Ophthalmological findings such as peripheral abnormalities of the retina or abnormal eye movements are sometimes observed. The majority of FCMD patients die during the second decade of life [Fukuyama et al., 1981].

Through positional cloning, we previously identified the gene responsible for FCMD, which encodes a novel 461-amino-acid protein that we have named fukutin (DDBJ/EMBL/GenBank AB008226) [Toda et al., 1993, 1996; Kobayashi et al., 1998a,b]. The *fukutin* gene spans a genomic DNA region of more than 100 kb on chromosome 9q31 and is composed of 10 exons [Kobayashi et al., 2001]. Most FCMD-bearing chromosomes (87%) are derived from a single ancestral founder, who lived 2,000–2,500 years ago and whose mutation consisted of a 3-kb retrotransposal insertion in the 3′ non-coding region of the *fukutin* gene [Colombo et al., 2000]. This insertion results in reduced *fukutin* mRNA levels. FCMD is the first known human disease to be caused by an ancient retrotransposal integration [Kobayashi et al., 1998a].

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Point mutations in the *fukutin* gene can render the FCMD phenotype rather severe, whereas the retrotransposon mutation is associated with a milder phenotype. All Japanese FCMD patients studied to date harbor at least one retrotransposon mutation [Kondo-Iida et al., 1999]. Only two Turkish patients with a more severe Walker–Warburg syndrome (WWS)-like phenotype have been identified with non-founder (point) mutations on both alleles [Beltrán-Valero de Bernabé et al., 2003; Silan et al., 2003], suggesting that most such genotypes are embryonic lethal and that fukutin is essential for normal development [Kurahashi et al., 2005]. Recent work suggests that fukutin is a glycosyltransferase involved in the modification of laminin-binding carbohydrate residues in α-dystroglycan [Toda et al., 2003].

Since the insertion is highly repetitive and GC-rich, Southern blot analysis has been used to detect it. Because this method is time-consuming and requires a considerable amount of DNA, however, it is unsuitable for prenatal diagnosis. A more rapid and efficient method for detecting the founder retrotransposal insertion would be highly useful for diagnostic tests and genetic studies.

The origins of the retrotransposal FCMD mutation remain unclear. 2,000–2,500 years ago, the Yayoi people migrated from Korea and China and intermingled with the indigenous Jomon people, generating the modern Japanese population. It is not known whether the founder FCMD mutation is a legacy of the migration or if the allele is unique to Japan.

Here we report the detailed sequence analysis of the founder retrotransposal mutation, a new method for rapid diagnostic detection of this mutation, and the distribution and origin of the founder mutation in Japanese and other Northeast Asian populations.

MATERIALS AND METHODS

Samples

We obtained a total of 2,814 DNA samples representing Japanese populations from various regions of Japan. We also analyzed DNA samples from Northeast Asia, including 935 Koreans, 203 Mongolians, and 766 Chinese (201 North Koreans, 197 North Hans, and 368 Manchurians), for a total of 4,718 DNA samples. Protocols in this study were approved by the Ethical Committee of Osaka University. These samples were treated as unlinked and anonymous.

DNA Isolation

Genomic DNA was prepared from peripheral blood leukocytes using the Nucleic Acid Purification System MFX-2000 (Toyobo, Osaka, Japan) with MagExtractor-Genome kit (Toyobo).

PCR

We performed a three primer-PCR method. Primer sequences were as follows: LAT7ura (forward, position 5,838), 5′-CCTTTACTCAAAAGATTGCATGACTGAATTT-GC-3′; LAT7-2 (reverse, position 5,994), 5′-AGTTCTATTGAGAGAAGGA-GGCAAACTGGTAA-3′; and ins 385–359 (reverse, position 340), 5′-TGAGCCGAGATGGCAGCAGCACCGTCC-3′. PCR was carried out in a final volume of 50 μl containing 1× LA PCR buffer II (Mg^{2+} free, Takara, Kyoto, Japan), 200 nM each primer, 160 μM deaza dGTP (Roche Diagnostics, IN), 160 μM dGTP, 320 μM other dNTPs, 20 ng of genomic DNA, 2.5 mM $MgCl_2$, and 2.5 U of Ex Taq DNA polymerase (Takara) using a GeneAmp PCR system 9700 (Perkin Elmer, Norwalk, CT). Cycling conditions were as follows: initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 sec and annealing-extension at 68°C for 3 min. PCR products were analyzed by electrophoresis on 2% agarose gels.

RESULTS

Composition of a 3-kb Retrotransposal Insertion Sequence Found in FCMD Patient

We determined the DNA sequence of a 3-kb retrotransposal insertion that was cloned from the genomic DNA cosmid library of an FCMD patient who was homozygous for the mutation. Sequence analysis revealed a 3,062-bp insertion enclosed by target-site duplications (a direct repeat of AAG-AAAAAAAAAATTGT) at both ends. The insertion contains hexanucleotide $(TCTCCC)_{41}$ repeats, GC-rich variable number tandem repeats (VNTRs) composed of 27 copies of a tandemly repeated 49-bp sequence (5′-GGGAGGGAGGTGGGGGGG-TCAGCCCCCCGCCTGGCCAGCCGCCCCATCC-3′), an *Alu* sequence, a polyadenylation signal (AATAAA), and a poly (A) sequence located at the 3′ end of the insertion. Figure 1 shows the complete insertion sequence (DDBJ/EMBL/GenBank AB185332) (see the online Fig. 1 at http://www.interscience.wiley.com/jpages/1552–4825/suppmat/index.html). This combination of sequence motifs is characteristic of a class of retroposons referred to as SINE-VNTR-*Alu* (SVA) [Shen et al., 1994].

Development of a Rapid PCR-Based Diagnostic Method to Detect a 3-kb Retrotransposal Insertion

We established a rapid PCR-based diagnostic method to detect the 3-kb retrotransposal insertion, as shown in Figure 2A. We used three primers (LAT7ura, LAT7-2, and ins 385–359) in one reaction mixture to detect both the normal and insertion alleles simultaneously. One primer pair (LAT7ura and ins 385–359) bridges the normal and insertion sequence, resulting in the amplification of a 375-bp product (lane 6, Fig. 2C). The second pair (LAT7ura and LAT7-2) generates a 157-bp product from the normal *fukutin* gene sequence (lanes 1–20 except 6, Fig. 2C). Since the insertion sequence is highly GC-rich, we used 7-deaza dGTP and a high denaturing temperature (98°C) to increase the specificity of the reaction and stabilize secondary structures [McConlogue et al., 1988]. Figure 2B depicts the use of this method to analyze an FCMD pedigree carrying a founder 3-kb retrotransposal insertion allele. Lanes 1, 2, 4, and 5 represent heterozygous carriers for the 3-kb insertion, as indicated by 375- and 157-bp bands. Lanes 3 and 6 show a normal individual and an insertion-homozygous FCMD patient, respectively. Thus, this method allows simultaneous detection of homozygous patients, heterozygous carriers, and normal individuals.

Distribution and Origin of the FCMD Founder Insertion in Japanese and Northeast Asian Populations

To investigate the distribution and origin of the 3-kb retrotransposal insertion, we used the rapid PCR detection method to screen a total of 4,718 genomic DNA samples from Japanese and other Northeast Asian populations, including Koreans, Mongolians, and Chinese (Fig. 3). Fifteen founder chromosomes were identified among 2,814 Japanese individuals. We found heterozygous carriers in various regions throughout Japan, including the Kinki and Kitakyushu areas, where the descendants of the migrated Yayoi people predominate, and the Okinawa and Aomori areas, where the influence of the indigenous Jomon people may be more prominent. Frequencies of the insertion in the various regions are as follows: Akita area (3 in 199), Kanagawa (1 in 105), Saga (1 in 139), Kitakyushu (1 in 178), Miyagi (1 in 203), Okinawa (1 in 205), Fukuoka (1 in 223), and Tokyo (3 in 785). Our analysis identified no FCMD carriers in the Aomori and Nagasaki districts.

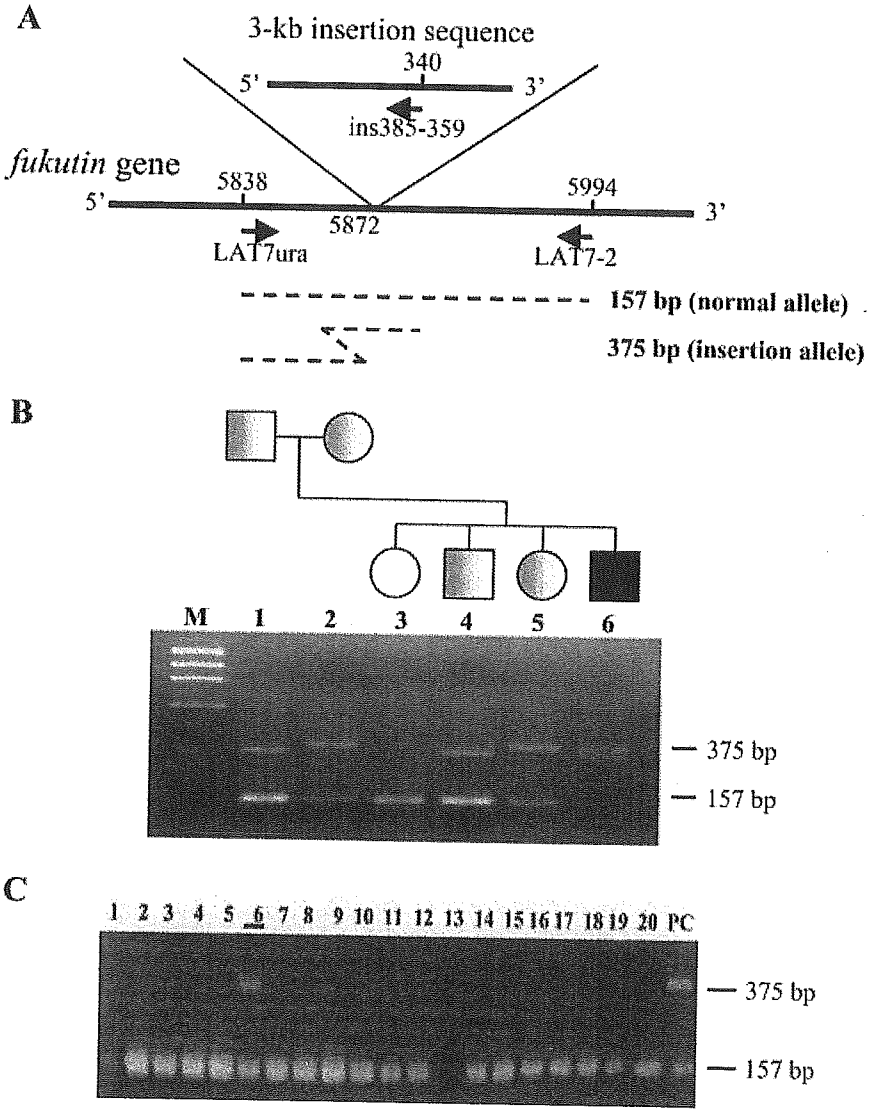

Fig. 2. **A**: Schematic representation of the rapid PCR-based method for detecting the 3-kb insertion sequence in Fukuyama-type congenital muscular dystrophy (FCMD). **B**: Pedigree of a family bearing the founder 3-kb retrotransposal insertion allele. **Lanes 1, 2, 4,** and **5** show heterozygous carriers, as indicated by PCR products of 375 and 157 bp. **Lane 3** shows a normal individual, as indicated by a 157-bp PCR product. **Lane 6** shows a FCMD patient who is homozygous for the insertion. **C**: Example of screening for FCMD carriers bearing the 3-kb insertion chromosome. Twenty genomic DNAs from Akita Prefecture were examined by PCR. Eighteen PCR products from **lanes 1** to **20** showed amplification of the normal 157-bp fragment only. **Lane 6** (underlined) indicates an individual bearing the 3-kb insertion. The sample in **lane 13** is not amplified. PC, positive control, a known compound heterozygous individual.

We detected one heterozygous carrier in 935 samples from Korean populations. However, we detected no heterozygous alleles in 203 Mongolians and 766 Mainland Chinese populations (201 samples from North Koreans, 197 samples from North Hans, and 368 samples from Manchurians). These data largely rule out the possibility that a single ancestor bearing an insertion chromosome immigrated to Japan from Korea or Mainland China with the Yayoi people approximately 2,000–2,500 years ago. However, we did detect a single insertion chromosome among 935 samples from Korean populations. This Korean carrier's family has lived near Seoul for several hundred years. The results obtained here estimate the carrier frequency of FCMD in the Japanese population to be 1 in 188 and appear to confirm that FCMD patients are rare outside of Japan.

DISCUSSION

FCMD, WWS, and muscle–eye–brain disease (MEB) are clinically similar autosomal recessive disorders characterized by congenital muscular dystrophy, cobblestone lissencephaly, and eye anomalies. FCMD is frequent in Japan, but only two FCMD patients with confirmed *fukutin* gene mutations has been identified in a non-Japanese population [Beltrán-Valero de Bernabé et al., 2003; Silan et al., 2003]. At present, the function of fukutin protein remains unknown. However, sequence analysis predicts fukutin to be an enzyme that modifies cell-surface glycoproteins or glycolipids [Aravind and Koonin, 1999]. This is supported by recent reports of a selective deficiency in highly glycosylated α-dystroglycan in FCMD, as well as defective glycosylation of α-dystroglycan in muscular

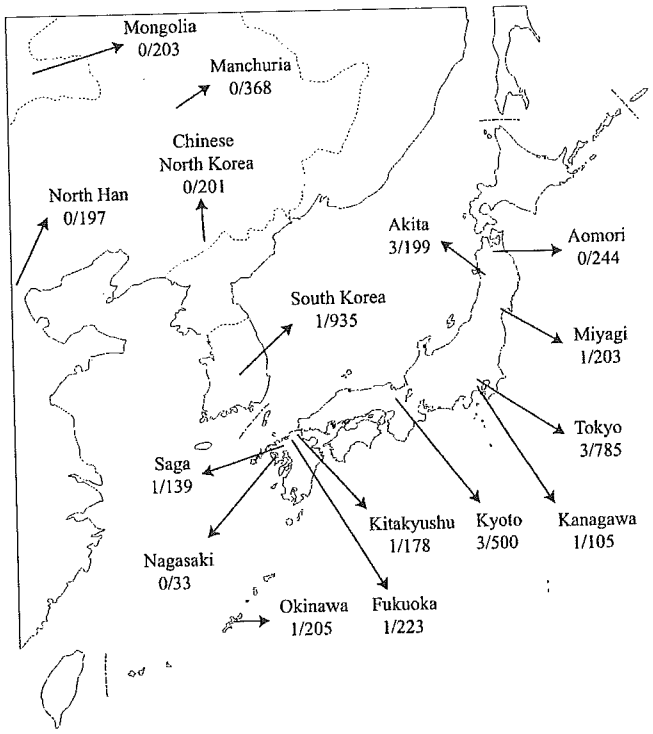

Fig. 3. Geographical distribution of FCMD carriers bearing the 3-kb retrotransposon insertion. The numerator indicates the number of FCMD carriers detected among the total sample, indicated in the denominator. Fifteen FCMD insertion carriers were detected in a total of 2,814 Japanese individuals, and one carrier was found in 935 Korean individuals. No insertion alleles were detected among 766 Chinese and 203 Mongolian individuals.

dystrophies caused by mutations in the putative glycosyltransferases, fukutin-related protein (FKRP), and mouse like-acetylglucosaminyltransferase (large) [Brockington et al., 2001; Grewal et al., 2001; Hayashi et al., 2001].

In addition, we have observed a selective deficiency of α-dystroglycan in MEB, which is caused by loss-of-function mutations in the gene encoding *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) [Yoshida et al., 2001]. A recent study found that 20% of WWS patients carry mutations in POMT1, a putative human counterpart of yeast *O*-mannosyltransferase [Beltrán-Valero de Bernabé et al., 2002]. Moreover, Michele et al. [2002] showed that in MEB, FCMD, and a mouse model of myodystrophy that α-dystroglycan is expressed in the muscle membrane, but similar hypoglycosylation in these diseases directly abolishes binding of dystroglycan to laminin, neurexin, and agrin. These findings suggest that defective glycosylation of α-dystroglycan due to genetic defects in glycosyltransferases may be the common denominator behind muscle cell degeneration in these diseases.

In previous studies, we have found that 87% of FCMD mutant alleles carry an retrotransposal insertion situated at position 5,872–5,873 in the 3′ untranslated region of the *fukutin* gene sequence [Kobayashi et al., 1998a]. Our sequence analysis has now identified this insertion as an SVA-retroposon.

The homozygous insertion of a 2,613-kb sequence similar to the 3-kb insertion in the *fukutin* gene was recently reported in an Italian patient (ARH7) with autosomal recessive hypercholesterolemia (ARH), an inherited form of hypercholesterolemia [Wilund et al., 2002]. The ARH7 proband has a SINE-VNTR-*Alu* (SVA) retroposon insertion in intron 1 of the ARH gene; the insertion is flanked by direct repeats (GAAACCTGTTTTCTC) and contains an *Alu* sequence at the 5′ end and a long terminal repeat (LTR) at the 3′ end. This insertion also contains multiple tandem copies of a 40–50 bp sequence characteristic of a VNTR sequence. The SVA sequence in the *ARH* gene was not detected in 35 normal individuals. SVA elements were first identified in the fourth intron of the RP1 gene, which was located immediately upstream of the complement *C4A* and *C4B* genes in the HLA class III region [Shen et al., 1994]. This sequence has yet to be fully characterized, and the mechanism by which it entered the ARH gene is currently unknown.

Recent morphological and genetic studies support the hypothesis that most of the population of contemporary Japan (Hondo-Japanese) are descended primarily from post-neolithic migrants who traveled from the East Asian continent in the Yayoi (300 B.C.–300 A.D.) and Kofun (300–600 A.D.) eras. The Ainu of Hokkaido (the northern island of Japan), as well as the Ryukyuans of Okinawa (southern islands of Japan), are thought to be descendants of preagricultural indigenous populations of Japan (Jomonese) [Hanihara, 1991; Bannai et al., 2000].

We have screened for heterozygous carriers of the founder 3-kb retrotransposal insertion in the *fukutin* gene in a total of 4,718 individuals from Japanese, Koreans, and Chinese populations. Previously, our group, along with Eberhart and Curran proposed the idea that a single ancestor bearing an FCMD insertion chromosome migrated to Japan with the Yayoi people, and that populations carrying the insertion have gradually increased through marriage with the indigenous Jomon population [Eberhart and Curran, 1998]. The data presented here largely rule out this hypothesis. However, this study did identify one South Korean individual who carried the insertion; its exact origin is unknown at present (Fig. 3). A Korean carrier might be originated from a migrant from Japan to Korea, since a considerable number of Japanese people migrated to Korea in 1500s and 1900s. This study suggests that an ancestor carrying the 3-kb retrotransposal insertion in the FCMD gene seems to belong to anyone of indigenous Japanese (Jomon people) populations, or a resident who migrated to Japan from Korean peninsula or Mainland China at the Jomon or Yayoi era and who already lived for years in Japan, since a negative geographic difference in incidences inside Japan has been seen in this study.

The results of the present study estimate the carrier frequency in the Japanese population to be 1 in 188, although previous reports have estimated the carrier frequency to be as high as 1 in 80 and the incidence to be 3–10/100,000 births [Fukuyama and Ohsawa, 1984].

A recent report showed a PCR-based method for a rapid detection of the insertion mutation [Kato et al., 2004]. The method needs two PCR reactions for one DNA sample. In our method, however, PCR is performed in one tube for one sample, where one of the primers is shared by both alleles of normal and insertion, and then one allele acts as an internal positive control of PCR for the other allele. Therefore, our diagnostic method described here will provide a more rapid and reliable diagnosis for FCMD that will be useful in genetic counseling and future genetic studies.

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