demonstrated this apparently paradoxical phenomenon in Fabry disease [4-7], and then in G_{M1}-gangliosidosis [9] and Gaucher disease [14]. The mutant protein expressed in the cell does not exhibit catalytic activity because of a defect in molecular folding and rapid degradation after biosynthesis [8,15]. This principle was recently demonstrated in a patient with Fabry disease with deficiency of α-galactosidase A by infusion of galactose for a short period [16].

We synthesized a new chemical compound NOEV as a potent inhibitor of human β-galactosidase [10], and anticipated that it would be useful for chemical chaperone therapy of patients with β-galactosidase deficiency. Our previous study confirmed stabilization and restoration of the enzyme activity by this chaperone compound in the G_{M1}-gangliosidosis model mouse expressing the R201C mutation [9].

In this study we tried a screening of patients with β-galactosidase deficiency for possible chaperone therapy using NOEV in the near future. Six cell strains in this study satisfied the two criteria for significant restoration of enzyme activity (3-fold increase and 10% of the control mean) to the level possibly sufficient for intraneural substrate degradation. We anticipate that the patients with the mutant genes satisfying one of two criteria in this study (at least 12% and at most 42%) will be good candidates for treatment and prevention of neurological manifestations during the course of the disease.

We postulate the lower limit of the enzyme activity for intracellular degradation of the substrates is 10 % of the control mean (54 nmol/h/mg protein) based on our previous cell and tissue experiments (unpublished data). However, there are a few cell strains, particularly from adult G_{M1}-gangliosidosis patients, with the residual enzyme activity already at this level. We are fully aware that the above working hypothesis is based on *in vitro* experiments using fibroblasts (not neural cells) and a synthetic (not physiological) substrate for enzyme assays.

A few common mutations are known to cause specific phenotypes, such as R428H and R208C for infantile G_{M1}-gangliosidosis, R201C for juvenile G_{M1}-gangliosidosis, I51T for

adult G_{M1}-gangliosidosis, and W273L for Morquio B disease [17-19]. In the present study the cells were collected randomly. However, the degree of efficacy in this study was dependent on the number of patients with common mutations causing individual phenotypes.

Under the conditions of our study, we found two different response types among the cells studied. Some cells responded to NOEV maximally at 0.2 μM and the others at 2 μM. This result indicates that the molecular interaction between the chaperone compound and mutant protein is mutation-specific. We anticipate that a molecular design will be possible for synthesis of new chaperone molecules for mutation-specific activity in future.

A similar therapeutic trial but in the opposite direction has been reported by inhibition of substrate biosynthesis, substrate deprivation therapy, for Gaucher disease [20] and G_{M1}-gangliosidosis [21]. In the latter using the disease model mice, ganglioside G_{M1} was reduced in the brain but asialo-ganglioside G_{A1} was not. More studies are necessary for solid conclusion on the biochemical and clinical effects of this trial.

The purpose of our study is to develop a new drug for G_{M1}-gangliosidosis, an intractable neurogenetic disease in children and adults. Chemical chaperone therapy has two major advantages over enzyme replacement therapy currently in use for medical practice: oral administration and accessibility to the brain [9]. Biosynthesis of a catalytically active enzyme is a prerequisite for chemical chaperone therapy. Although this new molecular approach is not efficient in all patients with a single lysosomal enzyme deficiency disorder, it is important that prevention or treatment could be achieved even in some of the patients with an intractable progressive neurological disorder.

Acknowledgments

This research was supported by grants from Ministry of Education, Culture, Science, Sports, and Technology of Japan (13680918, 14207106), and Ministry of Health, Labour and Welfare of Japan (H10-No-006, H14-Kokoro-017, H17-Kokoro-019).

References

[1] Suzuki Y, Oshima A, Nanba E. β-Galactosidase deficiency (β-galactosidosis): G_{M1}-gangliosidosis and Morquio B disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, editors, The metabolic and molecular bases of inherited disease, 8th ed. New York: McGraw-Hill; 2001: p.3775-3809.

[2] O'Brien JS, Storb R, Raff RF, Harding J, Appelbaum F, Morimoto S, et al. Bone marrow transplantation in canine G_{M1} gangliosidosis. Clin Genet 1990;38:274-280.

[3] Tylki Szymanska A, Maciejko D, Kidawa M, Jablonska Budaj U, Czartoryska B. Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. J Inherited Metab Dis 1985;8:101-104.

[4] Okumiya T, Ishii S, Takenaka T, Kase R, Kamei S, Sakuraba H, et al. Galactose stabilizes various missense mutants of α-galactosidase in Fabry disease. Biochem Biophys Res Commun 1995:214;1219-1224.

[5] Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal α-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. Nat Med 1999:5;112-115.

[6] Ishii S, Kase R, Sakuraba H, Taya C, Yonekawa H, Okumiya T, et al. α-Galactosidase transgenic mouse: heterogeneous gene expression and posttranslational glycosylation in tissues. Glycoconj J 1998:15;591-594.

[7] Okumiya T, Ishii S, Kase R, Kamei S, Sakuraba H, Suzuki Y. α-Galactosidase gene mutations in Fabry disease: heterogeneous expressions of mutant enzyme proteins. Hum Genet 1995;95:557-561.

[8] Ishii S, Kase R, Okumiya T, Sakuraba H, Suzuki Y. Aggregation of the inactive form of human α-galactosidase in the endoplasmic reticulum. Biochem Bophys Res Commun 1996;220:812-815.

[9] Matsuda J, Suzuki O, Oshima A, Yamamoto Y, Noguchi A, Takimoto K, et al. Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis. Proc Natl Acad Sci U S A 2003;100:15912-15917.

[10] Ogawa S, Kobayashi Matsunaga Y, Suzuki Y. Chemical modification of the β-glucocerebrosidase inhibitor N-octyl-β-valienamine: synthesis and biological evaluation of 4-epimeric and 4-*O*-(β-D-galactopyransyl) derivatives. Bioorg Med Chem 2002;10:1967–1972.

[11] Sakuraba, Aoyagi T, Suzuki Y. Galactosialidosis (β-galactosidase-neuraminidase deficiency): a possible role of serine-thiol proteases in the degradation of β-galacto-sidase molecules. Clin Chim Acta 1982;125:275-282.

[12] Tominaga L, Ogawa Y, Taniguchi M, Ohno K, Matuda J, Oshima A, et al. Galactonojirimycin derivatives restore mutant human β-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. Brain Dev 2001;23:284-287.

[13] Zhang H, Nanba E, Yamamoto T, Ninomiya H, Ohno K, Mizuguchi M, et al. Mutational analysis of TSC1 and TSC2 genes in Japanese patients with tuberous sclerosis complex. J Hum Genet 1999;44: 391-396.

[14] Lin H, Sugimoto Y, Ohsaki Y, Ninomiya H, Oka A, Taniguchi M, et al. N-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease. Biochim Biophys Acta 2004;1689:219-228.

[15] Zhang S, Bagshaw R, Hilson W, Oho Y, Hinek A, Clarke JT, et al. Characterization of β-galactosidase mutations Asp332->Asn and Arg148->Ser, and a polymorphism, Ser532->Gly, in a case of G_{M1} gangliosidosis. Biochem J 2000;348 Pt 3:621-632.

[16] Frustaci A, Chimenti C, Ricci R, Natale L, Russo MA, Pieroni M, et al. Improvement in cardiac function in the cardiac variant of Fabry's disease with galactose-infusion therapy. N Engl J Med 2001;345:25-32.

[17] Yoshida K, Oshima A, Shimmoto M, Fukuhara Y, Sakuraba H, Yanagisawa N, et al. Human β-galactosidase gene mutations in G_{M1}-gangliosidosis: a common mutation among Japanese adult/chronic cases. Am J Hum Genet 1991;49:435-442.

[18] Oshima A, Yoshida K, Shimmoto M, Fukuhara Y, Sakuraba H, Suzuki Y. Human β-galactosidase gene mutations in Morquio B disease. Am J Hum Genet 1991;49:1091-1093.

[19] Mosna G., Fattore S, Tubiello G, Brocca S, Trubia M, Gianazaa E, et al. A homozygous missense arginine to histidine substitution at position 482 of the β-galatosidase in an Italian infantile G_{M1}-ganglosidosis patient. Hum Genet 1992;90:247-250.

[20] Heitner R, Elstein D, Aerts J, Weely S, Zimran A. Low-dose N-butyldeoxynojirimycin (OGT 918) for type I Gaucher disease. Blood Cells Mol Dis 2002;28:127-133.

[21] Kasperzyk JL, d'Azzo A, Platt FM, Alroy J, Seyfried TN. Substrate reduction reduces gangliosides in postnatal cerebrum-brainstem and cerebellum in G_{M1} gangliosidosis mice. J Lipid Res 2005;46:744-751.

Figure Legends

Fig. 1. Inhibition of β-galactosidase activity by NOEV in control human fibroblasts. NOEV was added to the enzyme assay mixture at final concentrations up to 0.5 μM. Inhibition of enzyme activity was dose-dependent. Each value is the mean of triplicate assays. ♦: normal control; □: pathological control (dysostosis multiplex congenita).

Fig. 2. β-Galactosidase activity after incubation for 4 days with or without NOEV. In some cell strains the enzyme activity was enhanced by 0.2-2 μM NOEV in the culture medium. Each value is the mean of triplicate assays. Two peaks of maximal activity were observed. Normal control values: mean 538±230 nmol/h/mg protein; range: 220-1071 (n=19), and 10% of the control mean: 54 nmol/h/mg protein.

Table 1. NOEV effect and phenotype

Phenotype	Onset	Total	Positive response	
			> 3-fold	> 10%
G_{M1}-gangliosidosis	Infantile	31	10	2
	Juvenile	8	7	4
	Adult	7	0	4
Morquio B		3	0	0
Intermediate		1	0	0
		50	17	10

The fibroblasts were cultured in the medium containing 2 μM NOEV for 4 days, and the enzyme activity was assayed. The positive response was defined as a more than 3-fold increase (>3-fold), or as an increase up to more than 10% of the control mean (>10%). The background activity was 3-10% in adult G_{M1}-gangliosidosis, and the resulting relative increase was not high as compared to infantile or juvenile G_{M1}-gangliosidosis.

Table 2. NOEV effect and genotype

Mutation	Relative increase	Optimal NOEV concentration
R457Q	5-10 fold	0.2 μM
R201C, R201H	5-10 fold	2 μM
Q255H, V439G, Y57X, Y324C, others	2-6 fold	0.2-2 μM
I51T, W273L, others	0.5-1.2 fold	–

The fibroblasts were cultured in the medium containing 2 μM NOEV for 4 days, and the enzyme assay was performed. The relative increase was calculated as compared to the activity without NOEV in the culture medium. In the homozygous mutants, the NOEV effect was clearly and unambiguously concluded under the conditions in this study; such as R457Q, R201C, or R201H (positive), and I51T or W273L (negative). No definite conclusion was possible for the optimal concentration of NOEV on the other mutations, such as Q255H, V439G, Y57X, Y324C and others, because they were found as heterozygous with another known or unidentified mutation.

Figure 1

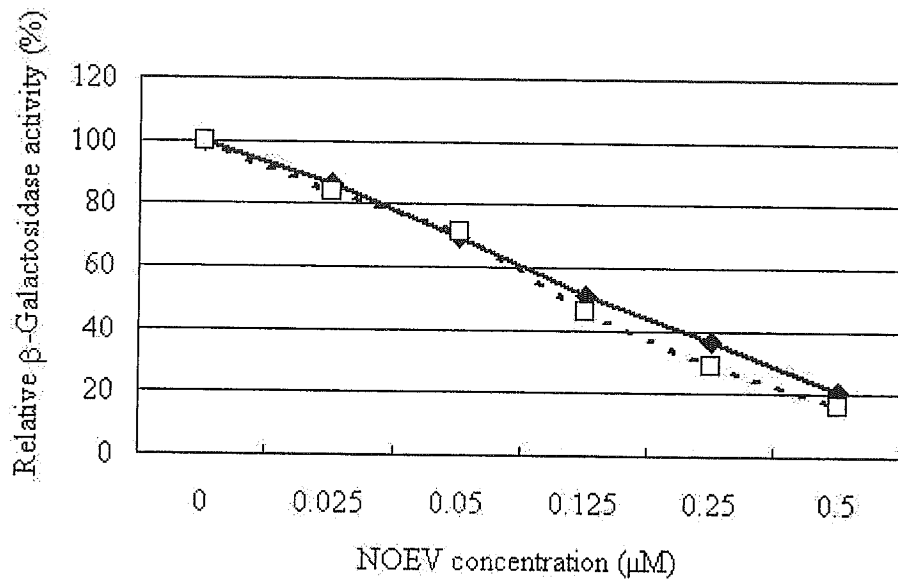
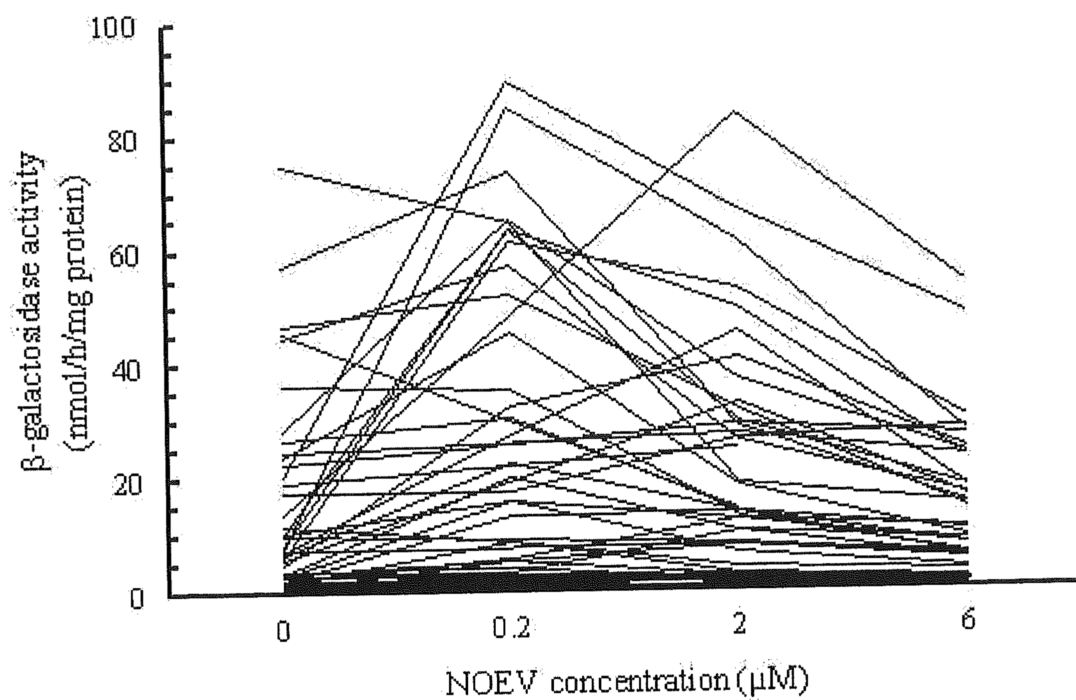


Figure 2



Six novel mutations detected in GALC gene in 17 Japanese patients with Krabbe disease and new genotype-phenotype correlation

Chengzhe Xu, Norio Sakai, Masako Taniike, Koji Inui, Keiichi Ozono

Department of Pediatrics,
Osaka University Graduate School of Medicine
2-2 Yamadaoka, Suita, Osaka 565-0871, JAPAN
Tel.: +81-6-6879-3935, Fax: +81-6-6879-3939
E-mail: norio@ped.med.osaka-u.ac.jp

For Peer Review

Abstract

Krabbe disease is an autosomal recessive leukodystrophy. It is pathologically characterized by demyelination in central and peripheral nervous systems, and the accumulation of globoid cells observed in brain white matter. It is caused by a deficiency of galactocerebrosidase (GALC) activity. We investigated mutations of the GALC gene in 17 Japanese patients with Krabbe disease, the largest subject number of Japanese patients to date, and found 27 mutations. Of these mutations, six were novel, including two nonsense mutations, W115X and R204X, two missense mutations, S257F and L364R, a small deletion, 393delT, and a small insertion, 1719-1720insT. Our findings, taken with the reported mutations in Japanese patients, confirm several mutations common to Japanese patients, the two most frequent mutations being 12Del3Ins and I66M+I289V, accounting for 37% of all mutant alleles. Along with two additional mutations, G270D and T652P, up to 57% of genetic mutations in Japanese patients may be accounted for. Distribution of the mutations within the GALC gene indicated some genotype-phenotype correlation. I66M+I289M, G270D and L618S contributed to a mild phenotype. Screening for these mutations may provide an effective method with which to predict the clinical phenotype.

Keywords Globoid cell leukodystrophy • Krabbe disease • Galactocerebrosidase •

Office, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Fax: +81-3-5449-543

Mutation analysis • Genotype-phenotype correlation

Introduction

Krabbe disease (globoid cell leukodystrophy: GLD, MIM 245200) is an autosomal recessive neurodegenerative disorder caused by the deficiency of galactocerebrosidase (GALC) (EC 3.2.1.46). It was first reported by Krabbe (1916), under the title, "A new familial, infantile form of diffuse brain sclerosis". Approximately 90% of patients exhibit the early infantile form, first manifesting symptoms before six months old and experiencing rapid disease progression over the ensuing one or two years (Wenger et al. 2001). The remaining 10% demonstrate late-onset Krabbe disease and are classified into one of three classes, late-infantile type, juvenile type or adult type, depending on the onset period and disease progression rate. Molecular cloning of the human GALC gene by Chen et al. (1993) (GenBank Accession No. L23116) and Sakai et al. (1994) (GenBank Accession No. L38544, L38559), has led to molecular-level analyses of Krabbe disease.

To date, the number of GALC mutations reported worldwide is more than 60, displaying molecular heterogeneity (Wenger et al. 1997). Whereas several papers (Tatsumi et al. 1995, Furuya et al. 1997, Kukita et al. 1997-98, Satoh et al. 1997, Fu et al. 1999) reported Krabbe disease mutations in Japanese patients, clear genotype-phenotype correlations remain obscure, due to the small number of subjects studied. We evaluated the GALC gene in 17 Japanese patients, classifying mutations as related to clinical phenotype. Here, we report the common mutations and the correlation between such mutations and their clinical severity.

Materials and methods

Patients

We studied 17 unrelated Japanese patients with Krabbe disease, originating from various regions of Japan. There were no consanguineous marriages between the patients' parents. The subjects included three (patients A1, A2 and A13) who were reported in a previous study, but were shown to have only one single mutant allele. The clinical information is summarized in Table 1. Diagnoses were determined in our laboratory by reduced GALC activity in either fibroblasts or leukocytes, and by other characteristic clinical and laboratory findings. According to the age at onset, patients were classified

Editorial Office, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Fax: +81-3-5449-5

into one of four clinical phenotype groups, including infantile onset: aged up to 6 months, 9 patients; late-infantile onset: 7 months to 2 years, 2 patients; juvenile onset: 3 to 8 years, 4 patients and adult onset: over 9 years, 2 patients.

Amplification of genomic DNA

After informed consent, genomic DNA was prepared from patients' peripheral blood leukocytes and/or cultured skin fibroblasts using standard methods, and entered into the subsequent studies. PCR reactions were carried out in 25 μl reaction volumes containing about 100 ng genomic DNA, 1XPCR reaction buffer (50 mM KCl, 10 mM Tris HCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, and 1.25 U Taq DNA Polymerase (Promega, Madison, USA). The thermal profile consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 51°C for 1min, and 72°C for 2 min, with a final extension at 72°C for 7 min. Five μl of each PCR mixture was run on agarose gel to make sure that only the specific product was amplified. Seventeen pairs of primer sequences for the amplication of exons and exon-intron boundaries of GALC gene are listed Table 2.

Screening for 12Del3Ins and I66M+I289V with restriction enzyme digestion

For the screening of 12Del3Ins (635-646del/insCTC resulting in 212-216 del (NLWES)/ins(TP)), a previously reported and relatively common mutation (Tatsumi et al. 1995; Fu et al. 1999), genomic DNA samples were amplified with the primer pair of exon 7 and the product was digested with *Hinf* I (Figure 1a). I66M+I289V, first reported by Furuya et al. (1997), is a unique mutation identified in the Japanese population to date. Only when two single-nucleotide substitutions (I66M, I289V) resided on the same allele, was their combination (I66M+I289V) proved to be a pathogenic mutation (Furuya et al. 1997). To detect I66M (198A>G), genomic DNA samples were amplified using a sense primer of exon 2 (Table 2) and a mismatch-antisense primer (5'-TCATTACCTTAAAGAGATAATCCGA-3'). The product was digested with *Eco* RV (Figure 1b). In order to detect I289V (865A>G), genomic DNA samples were amplified with a sense primer of exon 9 (Table 2) and a mismatch-antisense primer (5'-ACTAGCCACTAAATTCCAGTCGA-3') and product was digested with *Sal* I (Figure 1c). All the digested fragments were subjected to electrophoresis in 3% NuSieve 3:1 agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA). The PCR amplification of three fragments was performed under the same conditions described above. When both I66M and I289V were heterozygous in a patient, mutation analysis of

the patient's parents with screening for I66M and I289V was necessary to clarify whether I66M and I289V resided on the same allele. For all observed digestion patterns different from the normal control, the corresponding fragments were re-amplified for direct sequencing analysis.

Screening for the 30 kb large deletion mutation

For screening of the 30 kb deletion mutation, a previously reported and common mutation in Caucasians, genomic DNA samples were amplified using three primers according to the method described by Luzi et al. (1995).

Denaturing high performance liquid chromatography (DHPLC)

For patients A1-A11, all of the 17 exons and exon-intron boundaries were amplified by polymerase chain reaction (PCR) as described above. DHPLC analysis was performed with the WAVE DNA Fragment Analysis System equipped with a DNASep Column (Transgenomic Omaha, NE, USA). Prior to mutation analysis, the PCR products for each exon were denatured at 94°C for 5 min, followed by gradual re-annealing at 94°C to 25°C over 45 min to enable the formation of heteroduplexes. All samples were run at 3 different oven temperatures, listed in Table 2. PCR-amplified products exhibiting a heteroduplex profile were re-amplified and used for direct sequencing analysis.

Reverse -Transcription PCR and direct sequencing

For patients with no mutations or only one mutation by common mutation screening or DHPLC, GALC mutation analysis was performed by sequencing cDNA. Total RNA was extracted from cultured skin fibroblasts or lymphocytes, and first-strand cDNA synthesis was performed with MMLV reverse transcriptase (Gibco BRL) according to the manufacturer's recommendations. The coding region was amplified by PCR in two overlapping fragments from first-strand cDNA, as described elsewhere (Fu et al. 1999). PCR products were directly sequenced using BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) and 3730 DNA Analyzer (Applied Biosystems, Warrington, UK).

Screening for S257F and L364R in healthy individuals

For the screening of two novel missense mutations (S257F, L364R), PCR amplification of genomic DNA from 100 healthy individuals was performed with the primer pair for exon 8 and exon 10 (Table 2) and the product was digested with *Eco* 57I and *Aci* I, respectively. The normal allele of 257S should be digested by *Eco* 57I and the mutant allele of 364R should be digested by *Aci* I.

Editorial Office, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Fax: +81-3-5449-54

Results

All 27 mutations detected in this study are listed in Table 1. The analysis procedures for GALC mutation detection are described in detail below.

Screening for 12Del3Ins and I66M+I289V with restriction enzyme digestion

DNA isolated from peripheral blood leukocytes or cultured skin fibroblasts were tested for mutation detection. Screening for 12Del3Ins mutation with restriction enzyme digestion disclosed that five patients (A2, A5, A7, A12, A15), including one patient (A2) reported previously, were heterozygous. As a result of screening for I66M+I289V, I66M was found to be homozygous in one patient (A17), heterozygous in three patients (A12, A13 and A14), and I289V was found to be homozygous in A14 and A17, and heterozygous in A12 and A13. In patients 14 and 17, I66M and I289V were easily confirmed to reside on the same allele. To confirm that I66M and I289V reside on the same allele in patients A12 and A13, their parents were also analyzed for I66M and I289V. In A12 and A13, both I66M and I289V were proved to reside on the same allele inherited from the father of A12 and the mother of A13, respectively. I289V substitution without I66M in patient A14 was considered to be a polymorphism. These mutations were also confirmed through direct sequencing analysis.

Screening for the 30 kb large deletion mutation

Shortened PCR products from the deleted allele were not detected in any of our patients. The most frequent mutation in Caucasians was not found in our Japanese patients.

Mutation screening with DHPLC and mutation detection with direct sequencing

DHPLC analysis was performed in 11 patients (A1-11). Seventeen abnormal DHPLC elution profiles (A1: exon 4, 9; A2: exon 7; A3: exon 7; A4: exon 10, 12 and 17; A5: exon 7; A7: exon 5, 7 and 17; A8: exon 4; A9: exon 14, 15; A10: exon 9, 16; A11: exon 14) were found. The corresponding amplicons were characterized by direct sequencing. As a result, four polymorphisms (A7: IVS5+71G>A, A9: IVS14+60C>T, A4: 1254C>T (S418S) and A9: 1637T>C (I546T)) and fifteen mutations (A1: W115X, P302A; A2: R204X, 12Del3Ins; A3: R204X, I234T; A4: L364R, T652P; A5: 12Del3Ins; A7: 12Del3Ins, T652P; A8: 393delT; A10: P302A, L618S; A11: R515H) were identified in the above seventeen amplicons by direct sequencing. Within four polymorphisms, two (IVS5+71G>A and IVS14+60C>T) were novel intronic polymorphisms and the other two (1254C>T (S418S) and 1637T>C (I546T)) were exonic polymorphisms which were

For Peer Review

reported previously (Sakai et al. 1994, Kukita et al. 1997-98). Within fifteen mutations, eleven mutations were first identified with DHPLC except two mutations (A1: P302A; A2: 12Del3Ins) identified in the previous study (Tatsumi et al. 1995) and two mutations (A5, A7: 12Del3Ins) already detected in the screening step for 12Del3Ins. Four different novel mutations (W115X, R204X, L364R and 393delT) were found in this step.

Reverse -Transcription PCR and direct sequencing

Two mutations were identified in nine patients (A1, 2, 3, 4, 7, 10, 12, 13, 17) by the above screening methods. Only one mutation (12Del3Ins, 393delT, R515H, I66M+I289V and 12Del3Ins) was found in five patients (A5: 12Del3Ins; A8: 393delT; A11: R515H; A14: I66M+I289V; A15: 12Del3Ins). In the remaining three patients (A6, 9, 16), no mutations were found. To identify other mutations, mutation analysis by RT-PCR and direct sequencing were performed in eight patients (A5, 6, 8, 9, 11, 14, 15, 16), and three different mutations except the mutations detected using the above methods were detected in four patients (S257F heterozygous in A9, G270D heterozygous in A11, 1719-1720insT heterozygous in A14 and G270D homozygous in A16). In the patient A14, I289V is also confirmed on the same allele with the 1719-1720insT, however the I289V is considered as polymorphism, because the substitution of I289V without I66M is proven to be polymorphysm (Furuya et al. 1997) and the 1719-1720insT resulted in frame shift leading premature stop codon. Two mutations (S257F and 1719-1720insT) were novel. These mutations were also confirmed by the direct sequencing of genomic DNA.

Screening for S257F and L364R in healthy individuals

Of the six novel mutations (W115X, R204X, S257F, L364R, 393delT and 1719-1720insT), two missense mutations (S257F, L364R) were screened with restriction enzyme digestion. These mutations were undetected in 100 healthy controls.

Genotype-Phenotype correlation

The results of detected mutations are summarized in Table 1 with reference to reported mutations in Japanese patients. Table 1 includes other clinical information about the patients. Fourteen different mutations identified in this study were found in 27 detected alleles of 17 patients, including nine missense mutations (I66M+I289V, I234T, S257F, G270D, P302A, L364R, L618S, T652P), two nonsense mutations (W115X and R204X), one small deletion (393delT), one small insertion (1719-1720T) and one deletion/insertion (12Del3Ins). Of these mutations, six were novel (W115X, R204X, S257F, L364R, 393delT and 1719-1720insT). All GALC mutations in Japanese patients

Editorial Office, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Fax: +81-3-5449-54

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

with their frequencies, including those previously reported, are summarized in Table 3. The distribution of the clinical phenotype for each mutation is summarized in Table 4. For rare mutations, those detected less than twice, the genotype-phenotype correlation remains indeterminate; however, in rather common mutations, the tendency between genotype and phenotype was observed. The distribution of the phenotype for 12Del3Ins, T652P and R515H was observed mostly in infantile-type Krabbe disease, while I66M+I289V, G270H and L618S were detected mostly in the adult type and never in the infantile form of the disease. Concerning the regional distribution of patients, there was no specific region for each mutation.

Discussion

In this report we detected 27 mutant alleles in 17 patients. We found six novel mutations in the GALC gene in Japanese patients with Krabbe disease. Two, W115X and R204X, were nonsense mutations while 393delT and 1719-1720insT resulted in frame shifts. It was obvious that all four were pathogenic mutations. The final two, S257F and L364R were missense mutations undetected in 100 healthy controls. These two loci are well conserved in different species, including monkey, dog and mouse (Luzi et al. 1997). This evidence, when taken together, suggests that these two missense mutations may be considered causative of Krabbe disease.

In four patients, only one mutant allele was detected. As our screening method covered the coding region and exon-intron boundary, undetected mutations may lie outside the analyzed region such as promoter and enhancer regions. We also did not analyze large-scale genomic rearrangement, other than the screened 30 kb deletion.

As reported in the literature, mutations of the GALC gene in Krabbe disease exhibited great heterogeneity. In Caucasians, the 30 kb large deletion reported by Rafi et al. (1995) and Luzi et al. (1995) was widespread within the patient population, having a frequency of 40-50%. Other mutations followed heterogeneous patterning. As in Caucasians, to date, the examination of Japanese patients revealed 14 mutations with a very heterogeneous distribution. It was considered hard to screen the GALC mutation because there is no common mutation in Japanese patients; however, as our results show, several common mutations exist. The most common mutation in Japanese patients is 12Del3Ins with a 0.22 allele frequency. The second most frequent mutation, I66M+I289V, exhibits a 0.15 prevalence.

Editorial Office, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Fax: +81-3-5449-543

For Peer Review

To date, these two mutations have been identified only in Japanese patients. Moreover, these two mutations are unique and difficult to conceive as recurrently occurring mutations. They might have derived from founder; however, the parents' samples necessary for haplotype analysis were unavailable from most of the families.

Referring to Tables 1 and 4, the most common mutation (12Del3Ins) along with the two other mutations (T652P and R515H) in the homozygous state (B1, B2, A6 and B5) resulted in the classic infantile phenotype. The second most common mutation, I66M+I289V, contributed to late onset-type Krabbe disease, as the homozygous state of this mutation was found only in the adult type (patients A17 and B8), the mildest form of the disease, while the heterozygous state was detected in the juvenile or adult form (patients A12, A13, A14 and B9). This concordance strongly suggests that the existence of this mutation leads to the mild clinical phenotype. Since screening for this mutation might directly reveal a mild phenotype of Krabbe disease in Japanese patients, we propose a screening method using restriction enzyme digestion with PCR fragments for I66M (Figure 1b) and I289V (Figure 1c) as being viable toward that end. For the missense mutations, G270D and L618S, similar concordance is shown in Tables 1 and 4, demonstrating that it will be effective to screen this mutation to estimate the mild phenotype.

For most enzyme deficiency diseases, it is generally believed that there is a correlation between residual enzyme activity and clinical severity; however, this does not appear to be the case for Krabbe disease. The expression experiment did not always reveal better residual activity in late-onset patients. Harzer et al. (2002) analyzed the substrate specificity for several mutations and, while the G270D mutation lost enzymatic activity for galactocerebroside as its natural substrate, nearly normal activity for psychosine, its second substrate, was preserved. This paper supports that measuring enzyme activity with one substrate did not indicate an essential defect. It might be important to analyze the substrate specificity of L618S, I66M+I289V to elucidate genotype-phenotype correlation.

In a previous study, Furuya et al. (1997) investigated I66M+I289V allele expression, finding decreased enzymatic activity only when these two amino acid changes occurred on the same allele. We likewise confirmed that I66M and I289V occurred on the same strand and same peptide in all four patients.

That I66M and I289V reside on the same strand suggests that each amino acid contributes

Editorial Office, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Fax: +81-3-5449-54

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

to a different function such as reaction center or substrate binding. Structure analysis of crystalized protein might be important for future understanding of the mechanism of this mutation.

Figure legends

Fig. 1 Mutation detection for 12Del3Ins and I66M+I289V with restriction enzyme digestion. **a** Genomic DNA samples were amplified with the primer pair of exon 7; product was digested with *Hinf* I and subjected to 3% Nusieve gel. Fragments with 12Del3Ins were not digested with *Hinf* I. **b** For detection of I66M, amplified fragments using a sense primer of exon 2 and a mismatch-antisense primer (5'-TCATTACCTTAAAGAGATAATCCGA-3') were digested with *Eco* RV. **c** To detect I289V, amplified fragments with a sense primer of exon 9 and a mismatch-antisense primer (5'-ACTAGCCACTAAATTCCAGTCGA-3') were digested with *Sal* I

Acknowledgements This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan and the Health and Labor Science Research.

For Peer Review

References

Chen YQ, Rafi MA, de Gala G, Wenger DA (1993) Cloning and expression of cDNA encoding human galactocerebrosidase, the enzyme deficient in globoid cell leukodystrophy. Hum Mol Genet 2:1841-1845

Fu L, Inui K, Nishigaki T, Tatsumi N, Tsukamoto H, Kokubu C, Muramatsu T, Okada S (1999) Molecular heterogeneity of Krabbe disease. J Inherit Metab Dis 22:155-162

Furuya H, Kukita Y, Nagano S, Sakai Y, Yamashita Y, Fukuyama H, Inatomi Y, Saito Y, Koike R, Tsuji S, Fukumaki Y, Hayashi K, Kobayashi T (1997) Adult onset globoid cell leukodystrophy (Krabbe disease): analysis of galactosylceramidase cDNA from four Japanese patients. Hum Genet 100:450-456

Harzer K, Knoblich R, Rolfs A, Bauer P, Eggers J (2002) Residual galactosylsphingosine (psychosine) beta-galactosidase activities and associated GALC mutations in late and very late onset Krabbe disease. Clin Chim Acta

317:77-84

Krabbe K (1916) A new familial, infantile form of diffuse brain sclerosis. Brain 39: 74-114

Kukita Y, Furuya H, Kobayashi T, Sakai N, Hayashi K (1997-98) Characterization of the GALC gene in three Japanese patients with adult-onset Krabbe disease. Genet Test 1:217-223

Luzi P, Rafi MA, Wenger DA (1995) Characterization of the large deletion in the GALC gene found in patients with Krabbe disease. Hum Mol Genet 4:2335- 2338

Luzi P, Rafi MA, Victoria T, Baskin GB, Wenger DA (1997) Characterization of the rhesus monkey galactocerebrosidase (GALC) cDNA and gene and identification of the mutation causing globoid cell leukodystrophy (Krabbe disease) in this primate. Genomics 42:319-324

Rafi MA, Luzi P, Chen YQ, Wenger DA (1995) A large deletion together with a point mutation in the GALC gene is a common mutant allele in patients with infantile Krabbe disease. Hum Mol Genet 4:1285-1289

Sakai N, Inui K, Fujii N, Fukushima H, Nishimoto J, Yanagihara I, Isegawa Y, Iwamatsu A, Okada S (1994) Krabbe disease: isolation and characterization of a full-length cDNA for human galactocerebrosidase. Biochem Biophys Res Commun 198:485-491

Satoh JI, Tokumoto H, Kurohara K, Yukitake M, Matsui M, Kuroda Y, Yamamoto T, Furuya H, Shinnoh N, Kobayashi T, Kukita Y, Hayashi K (1997) Adult-onset Krabbe disease with homozygous T1853C mutation in the galactocerebrosidase gene. Unusual MRI findings of corticospinal tract demyelination. Neurology 49:1392-1399

Tatsumi N, Inui K, Sakai N, Fukushima H, Nishimoto J, Yanagihara I, Nishigaki T, Tsukamoto H, Fu L, Taniike M, Okada S (1995) Molecular defects in Krabbe Disease. Hum Mol Genet 4:1865-1868

Wenger DA, Rafi MA, Luzi P (1997) Molecular genetics of Krabbe disease (globoid cell leukodystrophy): diagnostic and clinical implications. Hum Mutat 10:268-279

Wenger DA, Suzuki Ku, Suzuki Y, Suzuki Ki (2001) Galactosylceramide lipidosis: Globoid cell leukodystrophy (Krabbe disease). In: Scriver CR, Beaudet AL, Sly WS,Valle D (eds) The metabolic and molecular bases of inherited disease.

Editorial Office, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. Fax: +81-3-5449-54