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ELSEVIER

Fibroblast screening for chaperone therapy in β-galactosidosis

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Abstract

We performed screening of β-galactosidase-deficient fibroblasts for possible chemical chaperone therapy using N-octyl-4-epi-β-valienamine (NOEV) in patients with G_{M1}-gangliosidosis and Morquio B disease (β-galactosidosis). Fibroblasts were cultured with NOEV for 4 days and β-galactosidase activity was measured. Mutation analysis was performed simultaneously. Two separate criteria were set for evaluation of the chaperone effect: a relative increase of enzyme activity (more than 3-fold), and an increase up to more than 10% normal enzyme activity. Among the 50 fibroblast strains tested, more than 3-fold increase was achieved in 17 cell strains (34%), and more than 10% normal activity in 10 (20%). Both criteria were satisfied in 6 (12%), and either of them in 21 (42%). Juvenile G_{M1}-gangliosidosis was most responsive, and then infantile G_{M1}-gangliosidosis. This enhancement was mutation-specific. We estimate that the NOEV chaperone therapy will be effective in 20–40% of the patients, mainly in juvenile and infantile G_{M1}-gangliosidosis patients. A molecular design may produce mutation-specific chaperone compounds for the other disease phenotypes. This cellular screening will be useful for identification of human patients with β-galactosidase deficiency for chaperone therapy to be started in the near future.
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Keywords: G_{M1}-gangliosidosis; β-Galactosidase; Gene mutation; N-Octyl-4-epi-β-valienamine; Chaperone therapy; Fibroblast

1. Introduction

Hereditary deficiency of lysosomal acid β-galactosidase (β-galactosidosis) causes two clinically distinct diseases in humans, G_{M1}-gangliosidosis and Morquio B disease [1]. The mode of inheritance is autosomal recessive. G_{M1}-gangliosidosis is a generalized neurosomatic disease occurring mainly in early infancy, and rarely in childhood or young adults. Morquio B disease is a rare bone disease without central nervous system involvement. Glycoconjugates with terminal β-galactose residues accumulate in tissues and urine from patients with these clinical phenotypes. Ganglioside G_{M1} and its asialo derivative G_{A1} accumulate in the G_{M1}-gangliosidosis brain. High amounts of oligosaccharides derived from keratan sulfate or glycoproteins are detected in visceral organs and urine from G_{M1}-gangliosidosis and Morquio B disease patients.

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At present only symptomatic therapy is available for human β-galactosidosis patients. Allogeneic bone marrow transplantation did not modify the subsequent clinical course or cerebral enzyme activity in a Portuguese water dog affected with G_{M1}-gangliosidosis [2]. Amniotic tissue transplantation was not effective in a patient with Morquio B disease [3]. Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis.

Recently we reported results of a molecular approach (chemical chaperone therapy) for restoration of mutant α-galactosidase in Fabry disease. Galactose and its structural analog, 1-deoxygalactonojirimycin, enhanced residual enzyme activity in cultured human lymphoblasts from patients with α-galactosidase deficiency [4,5], and transgenic mouse tissues expressing a mutant enzyme causing Fabry disease [5,6]. Some mutant proteins are unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus and are rapidly degraded without appropriate molecular folding [7,8]. Exogenously supplied chemical compounds that inhibit enzyme activity in vitro bind to the enzyme intracellularly to form a complex, stabilizing and transporting the catalytically active enzyme to lysosomes. The complex dissociates under the acidic condition in lysosomes, and the mutant enzyme remains stabilized and functional.

In a previous report we confirmed the effect of a new chemical compound *N*-octyl-4-epi-β-valienamine (NOEV) on cultured fibroblasts and model mice expressing a mutant β-galactosidase protein R201C [9]. In this study, we conducted screening of the patients with β-galactosidase deficiency for possible chaperone therapy in the near future.

2. Materials and methods

2.1. Chaperone compound NOEV

NOEV was synthesized by modification of a glucocerebrosidase inhibitor [9,10]. It is stable at room temperature and strongly inhibits human β-galactosidase in vitro. It is freely soluble in methanol or dimethylsulfoxide, and soluble in water up to 3–5 mM at room temperature. The molecular weight is 287.40.

2.2. Fibroblast culture

Fibroblasts from patients with β-galactosidase deficiency (G_{M1}-gangliosidosis or Morquio B disease) were stored in our laboratories, purchased from Coriell Cell Repositories (Camden, NJ, USA), or provided by the following colleagues at medical and scientific institutions: Mark Abramowicz and Patrick Van Bogaert (Brussels), Nils U. Bosshard (Zurich), Ernst Christensen

(Copenhagen), Fatih Süheyl Ezgü (Ankara), Mirella Filocamo (Genova), Agata Fiumara (Catania), Erentraud Irnberger (Salzburg), Koji Inui (Osaka), Wim J. Kleijer (Rotterdam), Jana Ledvinova (Prague), Gert Matthijs (Leuven), Toshihiro Oura (Sendai), Alan Percy (Birmingham, AL), Konrad Sandhoff and Gerhild van Echten-Deckert (Bonn), George H. Thomas (Baltimore, MD), David A. Wenger (Philadelphia, PA), and Marie-Therese Zabot (Lyon). The fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics, and harvested by scraping. They were collected by centrifugation, washed once with phosphate-buffered saline, and suspended in water. The cell suspension was sonicated, and used for enzyme assay (enzyme solution).

2.3. Enzyme assay

β-Galactosidase assay was performed on 96-well plates. The enzyme assay mixture consisted of 10 μl enzyme solution, with or without NOEV at the final concentration up to 5 μM, and 10 μl substrate solution containing 1 mM of 4-methylumbelliferyl-β-galactoside (Sigma, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5) and 0.1 M NaCl. After incubation for 1 h at 37 °C, the enzyme reaction was terminated by adding 0.2 M glycine–NaOH buffer (pH 10.7), and the liberated 4-methylumbelliferone was measured by fluorometry (excitation 355 nm; emission 460 nm) as described previously [11]. Protein was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.4. In vitro NOEV experiment

In this experiment human fibroblasts expressing normal (wild-type) β-galactosidase activity were used as an enzyme source, and NOEV was added to the enzyme assay mixture at final concentrations of 0–5 μM.

2.5. In situ NOEV experiments

Confluent fibroblasts (wild-type or mutant) were cultured in DMEM with or without NOEV (0, 0.2, 2, or 6 μM) on a 3.5-cm culture dish for a short-term experiment (4 days), or on a 10-cm culture dish for a long-term experiment up to 8 weeks. During the long-term culture the culture medium was changed regularly twice a week. Every 7–8 days, the cells were trypsinized, one-half was stored for enzyme assay and the other half was kept on culture.

For final harvesting and enzyme assay, the cells were scraped, collected by centrifugation, washed once with phosphate-buffered saline, suspended in water, and homogenized by sonication. The cell pellets were kept frozen at −80 °C until enzyme assay.

2.6. Gene mutation analysis

We tried to collect information about clinical and genetic data for each of the patients. However, information about phenotype and genotype was not always satisfactory. Enzyme deficiency was confirmed in our laboratory for all fibroblast strains in this study. Some cell strains without known genotype were subjected to gene mutation analysis [12]. After extraction of genomic DNA from human fibroblasts, each of the 16 exons with flanking sequence was amplified by polymerase chain reaction under the standard conditions. All exons except 1, 4, 7, and 9 were sequenced directly using ABI Prism 3100 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). The amplified exons 1, 4, 7, and 9 were subjected to single strand conformation polymorphism [13]. Exons with aberrant bands were subcloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

3. Results

3.1. In vitro NOEV experiment

Addition of NOEV resulted in a dose-dependent inhibition of the normal human β-galactosidase activity in vitro (Fig. 1). It was reduced to 20% of the background activity at the concentration of 0.5 μM in the assay mixture. The IC_{50} was calculated as 0.125 μM.

3.2. Time course of enzyme activity in cultured fibroblasts in response to NOEV

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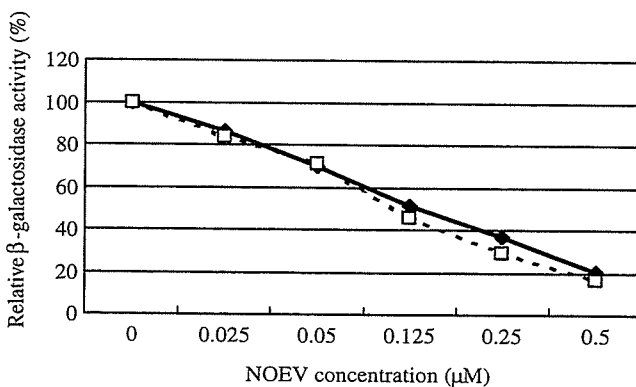
Fig. 2. β-Galactosidase activity after incubation for 4 days with or without NOEV. In some cell strains the enzyme activity was enhanced by 0.2–2 μM NOEV in the culture medium. Each value is the mean of triplicate assays. Two peaks of maximal activity were observed. Normal control values: mean 538 ±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

residual enzyme activities. In some cell strains, the enzyme activity was significantly enhanced after incubation for 2–4 days with 0.2–2 μM NOEV in the culture medium (Fig. 2). The increase continued up to 7 days and then remained at the same level for 8 weeks (data not shown). The rate of cell proliferation remained the same as that for the cells without NOEV treatment.

3.3. NOEV effect and phenotype

Table 1 shows the cumulative summary of the cell study. The positive response was defined either as more than 3-fold increase, or as an increase up to 10% or more of the control mean (54 nmol/h/mg protein). The first condition was satisfied in 17 cell strains (34%), and the second condition in 10 (20%); both conditions were satisfied in 6 (12%), and either of them in 21 (42%). The

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).
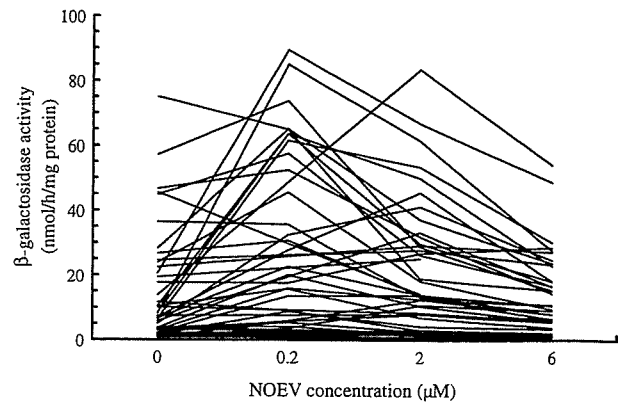
Table 1
NOEV effect and phenotype

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.

maximal enzyme activity was observed in two peaks either at 0.2 or 2 μM in most cell strains with positive response.

Juvenile G_{M1}-gangliosidosis was most responsive among the four clinical phenotypes tested in this study; relative increase in 7 of 8, and higher than normal 10% activity in 4 of 8. There was a relative increase of enzyme activity in 10 of 31 infantile G_{M1}-gangliosidosis cells in response to NOEV, but the enzyme activity reached more than the 10% normal level in only 2 cell strains. Adult G_{M1}-gangliosidosis and Morquio B disease apparently did not respond well to NOEV under the experimental conditions in this study. In general they showed relatively high residual enzyme activity, and the enzyme activity after NOEV treatment did not reach the 3-fold increase level, although an increase up to 10% of the normal control mean activity was achieved in four of seven cell strains.

3.4. NOEV effect and genotype

We collected more than 50 different β-galactosidase gene mutations [1]. In this study gene mutation analysis revealed several new or known mutations (data not shown). The effect of NOEV was genotype-specific (Table 2). Among the mutations examined, the amino acid substitution at 201 (R201C, R201H) causing juvenile G_{M1}-gangliosidosis responded maximally to NOEV at 2 μM, and the amino acid substitution at 457 (R457Q) causing infantile G_{M1}-gangliosidosis at 0.2 μM. The effect for these amino acid 457 or 201 mutations was confirmed in homozygous mutants. The response was less remarkable in compound heterozygotes with Q255H, V439G, Y57X, Y324C, or other mutations in human fibroblasts with β-galactosidase deficiency disorders (Table 2).

Table 2
NOEV effect and genotype

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

4. Discussion

Low molecular weight compounds for chemical chaperone therapy act as in vitro inhibitors at high concentrations and as in situ activators at low concentrations. We first 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

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Ⅶ. 薬物治療の新しい薬

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要 旨

ライソゾーム病の脳障害に対するケミカルシャペロン療法を開発中である. シャペロン化合物は酵素欠損症患者の細胞内で変異蛋白質分子を安定化し, 酵素活性を発現する. G_{M1}-ガングリオシドーシスモデルマウスへの経口投与により, シャペロン化合物 NOEV は血液脳関門を通り脳に到達し, 脳病変を改善させる. 治療法効果判定のためにマウスの神経学的検査法を確定し, NOEV の長期投与実験における臨床評価を実施中である.

Key Words

シャペロン
NOEV
G_{M1}-ガングリオシドーシス
β-ガラクトシダーゼ
分子治療

シャペロンとは

遺伝子病治療の最終目標は, 原因遺伝子を修復することである. しかし, 遺伝子異常は全身性であり, すべての細胞を治療することは不可能である. 多くは脳組織の原発性病変を発現する. われわれはライソゾーム病をモデルとして, 脳の遺伝病に発現される変異蛋白質の機能を修復させるために, 血液脳関門を通過する低分子シャペロンによる治療実験を始めた.

「シャペロン」は, 元来フランス語 chaperon であるが, 英国で, 貴族 (14世紀), 貴婦人 (16世紀) のかぶる頭巾・帽子, ガーター勲位の装束の一部 (16世紀), 棺を引く馬の前頭部につける飾り (17世紀), などの意味を経て, 若い未婚女性の社交界へのデビューに際し, 行儀作法を指導監督する年配の既婚女性 (18世紀), などの意味に使われた. 現在の臨床医学では, 医師が異なった性 (とくに女性) の患者を診察するときに医師に付き添う人 (女性), という意味で使われる. 現代英語では, chaperone という女性形単語として使われることが多い. 生物学では, 熱ショック蛋白質などの「分子シャペロン」として, 「他の蛋白質や蛋白質複合体の適正な折りたたみや構築を行う別の蛋白質」と定義される.

ライゾーム病の分子病態とその矯正

ライゾームは体細胞の小胞体である．数十の加水分解酵素が酸性の条件で高分子代謝産物を順序よく消化する．酵素遺伝子のひとつに変異がおこると酵素活性欠損，細胞機能障害，そして全身病として発現する（ライゾーム病）．多くは小児期の進行性中枢神経疾患である[1][2]．われわれは，とくに脳に特異的な脂質であるガングリオシドの分解酵素 β-ガラクトシダーゼの病態を詳細に調べてきた[3]．1988 年に酵素の責任遺伝子構造を解明後，多くの変異遺伝子を発見し，患者細胞内での変異蛋白質の動態を調べ，「酵素欠損」と総称される病気の分子病態が一様でないことを知った[3]〜[6]．つまり，蛋白質分子の合成障害，蛋白質分子の機能障害，蛋白質分子の細胞内不安定性，の3種である．

最初の二つの病態では，正常な酵素蛋白質あるいは遺伝子を補給しない限り，細胞の機能を正常化することは不可能である．しかし第三の場合，構造上活性をもつはずの酵素蛋白質が速やかに分解され，機能を発現できない．したがって，この蛋白質に適切な細胞内環境を提供すれば，働くべき場所で活性を復元できるかもしれない．

この論理をまず Fabry 病（α-ガラクトシダーゼ A 欠損症）の分子病態に適用し，変異酵素蛋白質を安定化させる化合物を投与することにより，実際に活性の発現誘導が実現した[7]．次に古典的な神経遺伝病 G_{M1}-ガングリオシドーシス（β-ガラクトシダーゼ欠損症）に対して脳の病態修復を試み，成功した[8]．
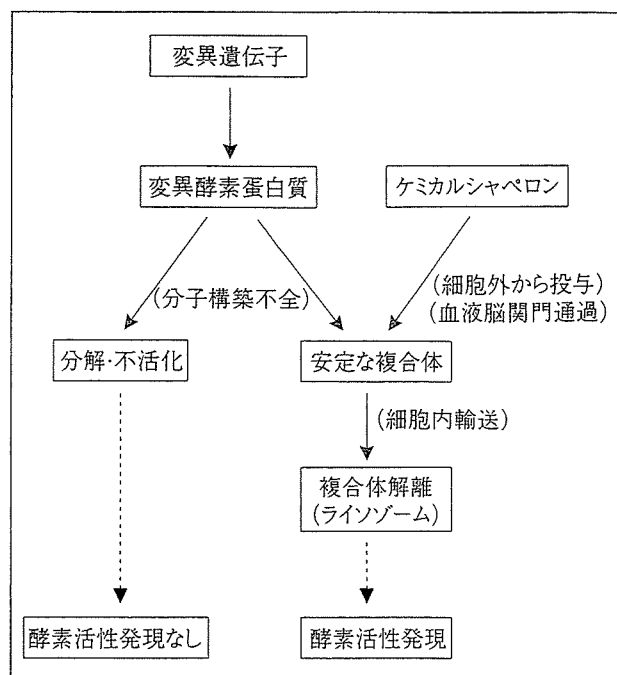
ケミカルシャペロン療法の原理

変異蛋白質分子の立体的な折りたたみ（フォールディング）が不十分なため，細胞内

図1 ライゾーム病に対するケミカルシャペロン療法の原理
投与した低分子シャペロン化合物は細胞内に入り，変異蛋白質と結合してその立体構造を修復し，細胞内輸送システムの働きによって無事にライゾームに運ばれる．ライゾームの酸性の環境で酵素分子とシャペロン分子の複合体は自動的に解離する．その結果，変異蛋白質は正常の構造を維持し，酵素としての活性を発現する．低分子化合物は経口投与後血流に入り，脳血管関門を通過して中枢神経系でシャペロン効果を示す

で速やかに分解，不活化される（つまり酵素活性が発現しない）ライゾーム病患者が存在する．一般に酵素分子に親和性の高い基質類似化合物が大量に試験管内に存在すれば，酵素の競合的阻害剤となる．ところが，細胞内にこの化合物が低濃度に存在する場合には，図1のような機構により，ライゾーム酵素の変異分子と安定な複合体を作り，ライゾームに輸送される．ライゾームでは酸性条件下で酵素分子が解離し，安定な触媒活性を示す．この種の化合物は，上記の分子シャペロンと本質的に同じはたらきをもつので，ケミカルシャペロン（chemical chaperone）とよぶことにする[8][9]．

この細胞処理で酵素の基質処理能力がある閾値以上にすれば，病気の発症を遅らせることができる．β-ガラクトシダーゼ欠損症の場合，活性が正常の 8 〜 10％になれば，理論上発症年齢
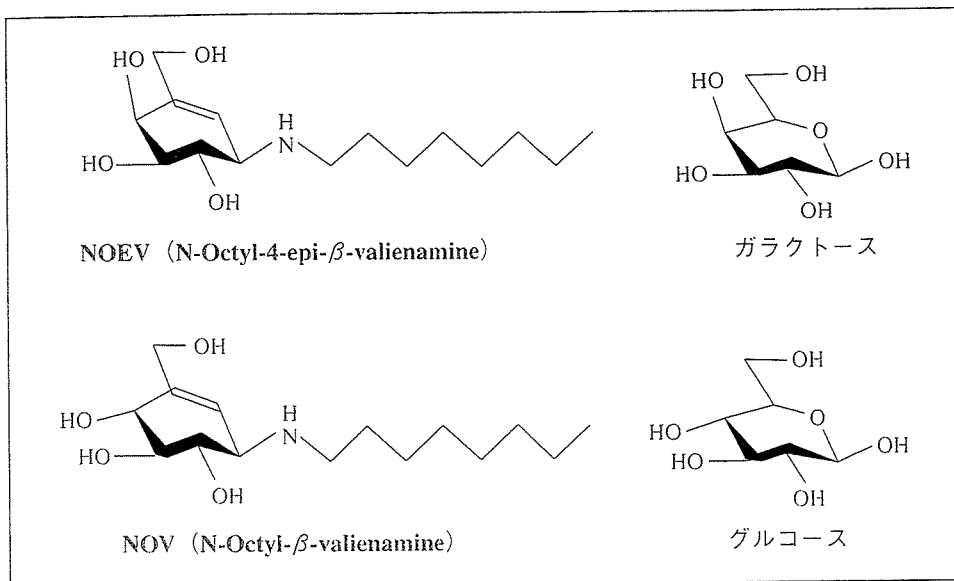
図2　NOEVとNOVの分子構造

NOEVはガラクトース，NOVはグルコース類似の化合物である．6環構造を持つがC1とC5を結ぶ
分子は酸素ではなく炭素である．そしてC1の炭化水素側鎖は窒素分子を介して結合している

が無限大となる．

ケミカルシャペロン療法には，患者細胞が触媒活性をもつ変異蛋白質を発現する，という条件が必須である．同じ名前の病気のすべての患者に適用できるわけではない．しかし現在，治療法のない病気の一部の患者でも，症状の軽減，予防が可能になれば，きわめて大きな学問的，社会的な意味をもつ．

新しいシャペロン化合物の検索とその成果

変異α-ガラクトシダーゼA分子の詳細な分析データをもとに，Fabry病患者細胞にガラクトースを投与したところ，活性が著しく増加した[10]．さらに，市販化合物，1-デオキシガラクトノジリマイシンがより低濃度で，この変異酵素にはたらくことがわかった[7]．

次にG$_{M1}$-ガングリオシドーシスを調べた[3]．1-デオキシガラクトノジリマイシンを試みたが，Fabry病と違い，β-ガラクトシダーゼに対する効果は，α-ガラクトシダーゼAの数十分の一であった[11]．次に新規合成化合物の酵素阻害ス

クリーニングを行った．その結果，NOEV（N-octyl-4-epi-β-valienamine）（図2）という化合物がβ-ガラクトシダーゼ活性の強力な阻害薬であることがわかった[8]．試験管内でのヒトβ-ガラクトシダーゼに対する50％阻害濃度は0.2 μMであった．この化合物は，類似化合物NOV（N-octyl-β-valienamine）のエピマーとして開発された化合物である[12]．NOVはβ-グルコシダーゼ阻害薬であり，一部のGaucher病患者細胞で活性発現が誘導される[13]．

シャペロン化合物NOEVの細胞内変異β-ガラクトシダーゼに対する効果

患者由来の線維芽細胞培養液にNOEVを添加して4日間培養すると，酵素活性が著しく上昇する細胞株があった．若年型症例の変異酵素R201Cにもっとも有効であり，乳児型症例にも有効な変異があった．すべての症例をあわせて35％の細胞が陽性反応を示した[14]．

すでに確立したβ-ガラクトシダーゼ欠損ノックアウト（KO）マウス[15][16]の線維芽細胞に

表　3種の遺伝子型マウスにおける神経学的評価総スコアの月別変移

月齢	2	3	4	5	6	7	8	9	10	11
WT	0.4	0.4	0.5	0.4	0.2	0.6	1.1	0.6	1	
Tg			4	4	3.8	3.7	3.3	6	7.9	11
KO				14	16	17	18	25		

WT：野生型マウス，Tg：トランスジェニックマウス（軽症型 G_{M1}-ガングリオシドーシス），KO：ノックアウトマウス（重症型 G_{M1}-ガングリオシドーシス）

R201C を導入した．ガングリオシド負荷後この細胞は脂質が著しく蓄積したが，NOEV が蓄積を著しく減少させた[8]．

遺伝子組み換えモデルマウスの作成と NOEV 治療効果

動物個体実験のために，KO マウスに R201C を導入したトランスジェニック（Tg）マウスを作製した[8]．正常な β-ガラクトシダーゼ遺伝子を導入したマウスは正常化した．つまりマウスで遺伝子治療ができたということである．R201C 変異を発現する Tg マウスは，KO マウスよりも緩徐な臨床経過を示す．発現する酵素活性は野生型マウスの 4％である[8]．実験的には，Tg マウスが軽症型，KO マウスが重症型の G_{M1}-ガングリオシドーシスモデルとなった．

この Tg マウスに NOEV 水溶液を経口投与し，脳を含むすべての組織の酵素活性上昇，脳組織の脂質蓄積消失を確認した[8]．脳内酵素活性は正常マウスの 30％，脳内 NOEV 濃度は肝臓内濃度の 30～45％であった（未発表データ）．この結果から，NOEV が腸管で吸収され，血液脳関門を通過して中枢神経系に到達し，酵素分子を安定化し，活性を発現させたとの結論を得た．

これまでのところ，この実験で体重，飲水量，血液生化学分析データに異常を認めていない．嗜好性や忌避はない．血液生化学，病理組織学的所見をさらに系統的に分析中である．

マウスの神経学的検査法の開発

NOEV の臨床効果を知るために，マウスの神経学的検査法を開発した．マウス脳の局在診断は困難であり，ヒト乳幼児の神経学的診察法をマウスに適用した．自発運動，個体各部位の姿勢肢位，原始反射，姿勢反射，平衡反応など，合計 11 項目をセットとした評価法を確立した（未発表データ）．それぞれの検査項目を 4 段階に評価し，最終的に合計点で総合評価を行った．

表に，3種の遺伝子型マウスにおける総スコアの時間的推移を示す．大まかな臨床観察では捉えられない初期変化を数字として表現することができた．加齢とともに WT マウス（野生型），Tg マウス（軽症型），KO マウス（重症型）の重症度の差が明確になった（投稿中）．さらに多数例についての時間経過観察，NOEV 投与の臨床評価が進行中である．

他のライソゾーム病・他の遺伝病への応用

現在の主要な研究対象は β-ガラクトシダーゼ欠損症（G_{M1}-ガングリオシドーシス）であるが，この新しいアプローチはすべてのライソゾーム病に適用できるはずである．NOEV は試験管内で，Krabbe 病欠損酵素の強い競合的阻害薬でもある．しか細胞実験で，欠損酵素の活性復元効果がまだ確認できていない．β-グルコシダーゼ欠損症（Gaucher 病）にも，NOV の有効性が培養細胞で確認された[13]．

われわれはライソゾーム病という，細胞内分子病態解析がかなり進んだ疾患群を対象とした研究を行ってきた．ほかの遺伝病でも変異遺伝子の発現，変異蛋白質の分子修飾，活性発現部位への細胞内輸送，活性発現機構などが明らかになれば，ケミカルシャペロン療法が可能であるはずである．

今後，多くの種類の遺伝病についての研究が発展することを期待している．

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Ⅲ．診断の進歩

遺伝子診断の実際

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要　旨

　遺伝子診断は，技術の進歩により出生前診断を含め先天代謝異常症の分野でも広く普及してきている．われわれは，WAVEシステムを用いたシステムを構築しており，GM1-ガングリオシドーシスを例に遺伝子診断の方法について述べる．倫理面では「遺伝学的検査のガイドライン」などを十分に配慮する必要がある．将来的には，DNAマイクロアレイなどの技術により多くの疾患の診断を，迅速に行うシステムが構築される可能性がある．

はじめに

　先天代謝異常症では，従来，蓄積物質の同定や酵素活性の測定によって診断がなされてきたが，近年，多くの原因遺伝子が明らかにされ，その遺伝子診断が確立されてきた．遺伝子診断は，解析技術の進歩により，現在では，比較的簡便に行うことができ，広く普及してきている．ゲノムDNAを用いるために，末梢血をはじめすべての臓器で検査可能であり，出生前診断にも利用されている．遺伝子診断はPCR法により，目的のDNA部分を増幅し，塩基配列の決定により異常を検出することで行われるが，解析する領域が多い場合には，塩基配列の決定とその情報を処理することが大変になる．そのために，変異部位をスクリーニングする方法を導入する場合も多い．

　われわれは，現在広く普及しているWAVEシステムを導入することにより効率的な遺伝子診断法を確立しているいる．

　本稿では，GM1-ガングリオシドーシスの遺伝子診断の例を示す．さらに，変異データの解釈，検体，倫理面，検査施設，最後に今後の解析技術についても述べる．

解析の具体例

従来，遺伝子変異検索には SSCP 法（single-strand conformation polymorphism）が汎用されてきた[1]．近年，低コストで効率よい DHPLC 法（denatured high-performance liquid chromatography：イオン対逆相-変性高速液体クロマトグラフィー）が SSCP 法に代り普及してきている[2]．

原理は，以下とおりである．正常型 DNA と変異型 DNA の変異を含む領域を PCR で増幅後，PCR 産物を 95℃で加熱してから，ゆっくりと冷却し再重合させ，2 種類のヘテロ二重鎖と 2 種類のホモ二本鎖を形成させる．変異のある部位ではミスマッチのため，水素結合が形成されないために水素結合が形成されず，カラムへの結合力が弱くなり，溶出時間の差として検出することができるというものである（図1）．フラグメント分離用のカラムと HPLC を組み合せた装置として，WAVE™ DNA Fragment Analysis System（Transgenomic, Inc.）が市販されている．

以下に，われわれが解析した WAVE システムを用いた β-ガラクトシダーゼ遺伝子変異解析について述べる．

ヒト β-ガラクトシダーゼ遺伝子（GLB1）は，3 番染色体3q21.33 領域に位置し，16 エクソン，2,031 塩基をコードしている．これら 16 のエクソン・イントロンジャンクションを含む領域が増幅される PCR プライマーを設計した（表1）．これらプライマーを用い，ヒト患者血液および皮膚繊維芽細胞より抽出したゲノム DNA を検体とし Ampli Taq Gold（Applied Biosystems）を用いた 95℃ 5 分，95℃ 1 分，60℃ 1 分，72℃ 1 分（35 サイクル），72℃ 5 分のサイクルで PCR 反応を行った（Gene Amp PCR System 9700, Applied Biosystems）．PCR 産物の一部をアガロースゲル電気泳動で確認後，サーマルサイクラーにて 95℃ 4 分間加熱後 45 分間かけて
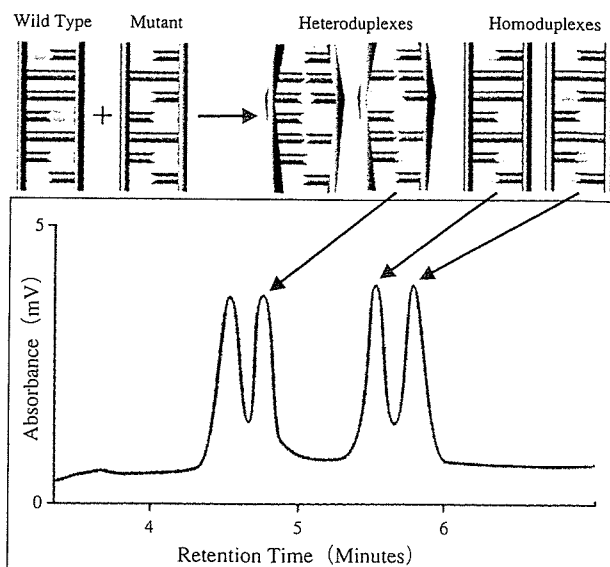

図1　DHPLC 法による変異解析の原理
http://www.transgenomic.com/より引用
変異を含む PCR 産物と正常 PCR 産物を混合し加熱再重合することでヘテロ二重鎖とホモ二重鎖を形成させ，HPLC で分離することにより異なる複数のピークが検出できる

25℃までアニーリングさせ，ヘテロ二重鎖の形成を行った．二重鎖の形成は正常型コントロール DNA，患者 DNA および正常と患者 DNA の混合液の 3 セットについて行い，DHPLC で解析した．

DHPLC 解析の変異検出感度を作用するのは，分離の温度 Tm および溶離液のグラジエントなどの分析条件の設定であるが，Tm は付属するソフトウェア（WAVEMAKER™ software：Transgenomic, Inc.）と，the Stanford Genome Technology Center software（**http://insertion. stanford.edu/melt.html**）の両方を用い，計算された Tm 値および上下 2℃の三点でのスクリーニングを行った．WAVE fragment 解析装置では，オートサンプラーにより 1ul のサンプルが分離カラムに注入後，DNA がイオン対逆相変性高速液体クロマトグラフィーにより分離される．検出は 254 nm における吸光度により行われる．正常型 DNA のみの場合，ホモ二本鎖の 1 本のピークが検出されるが，サンプル DNA に変異または多型が存在する場合，正常型 DNA とは異なる 2 本，もしくはそれ以上のピークと

して検出される.

最後に変異のピークが見られた産物については，直接シークエンス法（ABI Prism 3130xl DNA Seqencer；Applied Biosystems）により遺伝子変異の同定を行った（図2）. これまで，13人のβ-ガラクトシダーゼ欠損症の患者について変異解析を行った結果11人について20の変異を同定し，そのうち9種類が新規変異であった（表2）.

WAVE装置の解析時間は1検体あたり10分程度と迅速であり，WAVE解析装置の価格はSSCP法に比べ高価であるが，導入できれば迅速，簡便かつ低コストな方法である.

変異データの解釈

0.1％のゲノム配列はすべてのヒトで異なっており，見つかった遺伝子の変異が疾患の原因にあたるかどうかを十分に考慮する必要がある. 理想的には発現実験などにより，遺伝子変異が遺伝子産物（蛋白）の機能異常をきたすことを証明することが理想である. しかし，これは臨床診断として用いるには現実的ではない.

通常は，インターネット上で利用できるデータベースとの照合を行い，変異であるかどうかを確認する. 正常多型のデータベースとしてはNCBIのSNPデータ（**http：//www. ncbi. nlm. nih. gov/ SNP/**）があり，遺伝子変異のデータ

表1　ヒトβ-ガラクトシダーゼ遺伝子に対するPCRプライマーとPCR増幅産物のサイズ

| exon	sense $(5'\!\rightarrow\!3')$	antisense $(5'\!\rightarrow\!3')$	PCR size (bp)
1	caggccgtgggtccttagtcaagt	gccagcctgtcccctagcaatg	204
2	gctactctcaaaggatcggcttctgaaa	tatcttctctccagagtgggtgttcagg	303
3	gccttctccctcttatccatgtgttagc	taaaagacacctgtgctgggtacagtcc	401
4	ccccttgtcccttgaagctttattctt	tgtatttttagtaggggcgaggttttgc	347
5	agtttacgaatttgtgttgggccacatt	gccttcccaaatgcaattgaactaaaag	358
6	aggatctcctcatttttccctgctcttt	atgaaaaatctcaatctgcccatgacac	330
7	actaacattctgaccgtagcaggcttc	tcattcacatgtccagaatggctatgac	325
8-9	ctttacacctgtcatagatggggcattg	cacacccctcctcaaattaatcaacaga	412
10	cgtctgtgtctcccaacaagtggttta	gtgagttcaaaagaggctctgtccaaga	312
11	gcactgttgagtctttgaccttgctttc	ttcgcagaaaaataacgaaccaattcct	301
12	gggagtagatggagaggactgaaggaga	ggatctgatgcatttgcttaccattttg	391
13	ggaggtggaggaagattttcattcctta	ctgaaaaggtgagcaaagaccccaaat	343
14	tctccttgctgaccttcttaccctcaat	tattttacccaggctggtcttgaactcc	348
15	atttcgaggttcatttcctgttggtgtt	aagtttaggcctgaattcaaacccttcc	427
16	ggggttgatggttctctgtctctctctc	gaaacctcaggtgaaaatgcacatccta	467
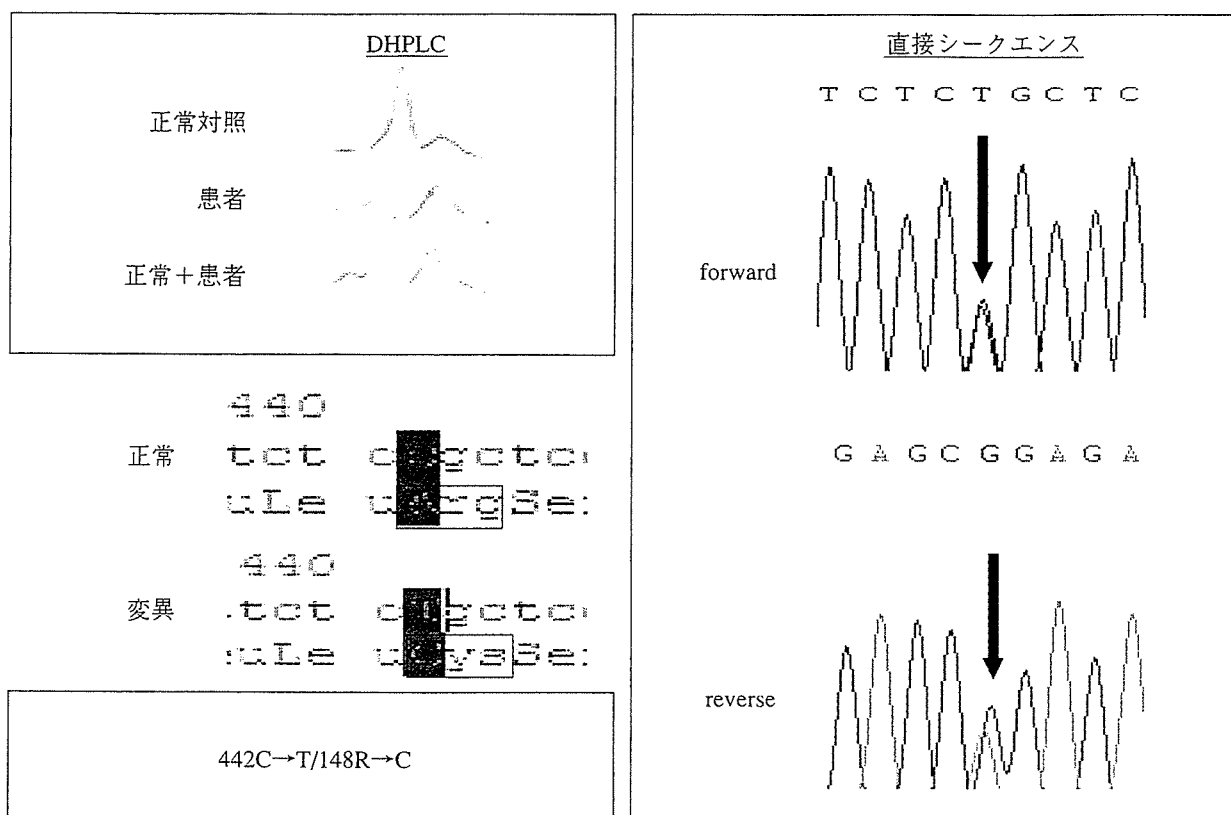
図2 ヒトβ-ガラクトシダーゼ遺伝子の DHPLC 変異解析の結果

DHPLC 解析により正常とは異なるパターンが得られる．ホモ接合体異常の場合を考慮して，正常対照，検体，検体＋正常の3セットのサンプルを検討する．最終的には直接シークエンスが変異を確認する

表2 ヒトβ-ガラクトシダーゼ遺伝子解析の結果（MMBID より一部引用）

NS	mutation	exon	amino acid	base		phenotype	note
1	I181K	5	181Ile → Leu	542T → A	Hm	Turkish, Inf	GM new
2	D640E	16	640Asp → Glu	1920C → G	ht	Japanese, Inf GM1	new
	M480V	14	480Met → Val	1438A → G	ht		new
	R482H	14	482Arg → His	1445G → A	ht		～ Italian Inf GM1, Caucasian MB
3	no mutation in all exons					Japanese, Inf GM1	
4	no mutation in all exons					Japanese, Inf GM1	
5	R148C	4	148Arg → Cys	442C → T	ht	Japanese, Inf GM1	new
6	R208C	6	208Arg → Cys	622C → T	Hm	Japanese, Inf GM1	～ American Inf GM1
7	R59H	2	59Arg → Cys	176G → A	ht	Japanese, Inf GM1	～ Brazilian Inf GM1
	D332E	10	332Asp → Glu	996C → G	ht		new
8	R482H	14	482Arg → His	1445G → A	ht	Japanese, Inf GM1	～ Italian Inf GM1, Caucasian MB
	P549L	15	549Pro → Ley	1646C → T	ht		new
9	276-277 ins G	3	frame shift + stop codon		Hm	Japanese, Inf GM1	new
10	R201C	6	201Arg → Cys	601C → T	ht	unknown	～ Japanese Juv. GM1
	R201H	6	201Arg → Hys	601C → T	ht		～ Caucasian Adult GM1
	R201Y	6	201Arg → Try	601-602CG → TA	ht		new
11	S54I	2	54Ser → Ile	161G → T	ht	unknown	new
12	R59C	2	59Arg → Cys	175C → T	ht	unknown	new
	T82M	2.3	82Thr → Met	245C → T	ht		～ Caucasian Adult GM1
13	R201C	6	201Arg → Cys	601C → T	ht	Jap Adult GM1	～ Japanese Juv. GM1
	T420K	13	420Thr → Lys	125C → A	ht		new

NS：Number of Patient Sample, Hm：homozygous, ht：heterozygous

ベースとしては The Human Gene Mutation Database（HGMD）（**http://www.hgmd.cf.ac.uk/ac/search.html**）などが存在し，これらのデータベースや過去の論文の記載などを参考にして，既知変異，新規変異，正常多型などの判断を行う.

検体に関して

遺伝子解析の材料としてはRNAとDNAが従来用いられてきた．現在では，DNAを解析することが主流となっている．一般的には末梢血を使うことが多いが，毛髪，頬粘膜，病理検体からのDNAを分離することが可能である．末梢血の場合は，EDTAを抗凝固薬として用いることが多い．4℃または室温でも数日は保存が可能であり，1〜5 ml 程度の採血で通常は十分なDNAが得られる.

倫理的な問題

遺伝子解析研究においては，「ヒトゲノム・遺伝子解析研究に関する倫理指針」（文部科学省，厚生労働省，経済産業省　平成13年3月29日，平成16年12月28日全部改正，平成17年6月29日一部改正）が定められており，各施設での倫理委員会の承認を得て行う必要がある．しかし，現在は研究目的よりも臨床の診断として日常的に遺伝子診断を行うことが多くなってきている．この場合には，「遺伝学的検査に関するガイドライン」（平成15年8月　遺伝医学関連10学会）に従って，十分な遺伝カウンセリング，書面によるインフォームド・コンセント，検体の匿名化などに留意し，遺伝子診断を進めていく.

遺伝子診断の検査施設に関して

そのほとんどが稀少遺伝病と考えられる先天代謝異常症においては，検査会社レベルでのコマーシャル検査の普及はむずかしく，研究室に頼らざるをえない状況にある．これらに対して，日本先天代謝異常学会のホームページ（**http://www.jsimd.org/**）上の「各施設の専門分野」，また京都大学で構築されている「ヒト Germline 遺伝子・染色体検査オンラインデータベース」（**http://www.kuhp.kyoto-u.ac.jp/idennet/DB/index3.html**）などが参考になる．さらに Gene Tests（**http://www.genetests.org/**）のサイトを利用することによりアメリカなどの検査施設の情報を得ることも可能である．しかし，遺伝子診断は，大学での研究としては成り立たなくなってきており，その遺伝子診断システムをどうするかは，今後の重要な課題である.

今後の解析技術について

われわれは，近年 WAVE システムを用いた解析技術を用いているが，DNA マイクロアレイ技術（DNA チップ技術）の進歩には目をみはるものがある．最近開発された Genome Tiling Array[3] などを利用することにより，多くの遺伝子の変異を一度に見つけることが可能になってきた．将来的には先天代謝異常症の遺伝子異常を，一度に簡便にスクリーニングできる DNA チップの開発が行われる可能性がある.

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第 10 回乳幼児けいれん研究会のお知らせ

会　　期　　2007（平成 19）年 4 月 7 日（土曜）〜 8 日（日曜）
会　　場　　慶應義塾大学三田キャンパス北館（東京）TEL 03-3453-4511
主　　題　　「乳幼児におけるけいれん準備性の生物学」
会　　長　　高橋孝雄（慶應義塾大学小児科教授）
公 用 語　　英語（国際シンポジウム形式）
早期登録締切　2006（平成 18）年 11 月 30 日
出題抄録締切　2006（平成 18）年 11 月 30 日
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Six novel mutations detected in the GALC gene in 17 Japanese patients with Krabbe disease, and new genotype–phenotype correlation

Abstract Krabbe disease is an autosomal recessive leukodystrophy. It is pathologically characterized by demyelination of the central and peripheral nervous systems and the accumulation of globoid cells 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 being 12Del3Ins and I66M + I289V, which account for 37% of all mutant alleles. With two additional mutations, G270D and T652P, these account for up to 57% of genetic mutations in Japanese patients. 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 · Mutation analysis · Genotype–phenotype correlation

Introduction

Krabbe disease (globoid cell leukodystrophy: GLD, MIM 245200) is an autosomal recessive neurodegenerative disorder caused by a 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 have the early infantile form, first manifesting symptoms before 6 months old and experiencing rapid disease progression over the ensuing 1 or 2 years (Wenger et al. 2001). The remaining 10% have 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 the rate of progression of the disease. 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.

More than 60 GALC mutations, all with molecular heterogeneity, have been reported worldwide (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) have reported Krabbe disease mutations in Japanese patients, clear genotype–phenotype correlations remain obscure, because of the small number of subjects studied. We evaluated the GALC gene in 17 Japanese patients, classifying mutations according 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 different regions of Japan.

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There were no consanguineous marriages between the patients' parents. The subjects included three (patients A1, A2, and A13) included in a previous study who were shown to have only one single mutant allele. The clinical information is summarized in Table 1. Diagnoses were determined in our laboratory as 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 into one of four clinical phenotype groups: infantile onset, aged up to 6 months, nine patients; late-infantile onset, 7 months to 2 years, two patients; juvenile onset, 3–8 years, four patients; and adult onset, over 9 years, two patients.

Amplification of genomic DNA

After informed consent, genomic DNA was prepared, by standard methods, from patients' peripheral blood leukocytes and/or cultured skin fibroblasts and used for the subsequent studies. PCR reactions were conducted in 25-μL reaction volumes containing approximately 100 ng genomic DNA, 1×PCR reaction buffer (50 mmol L^{-1} KCl, 10 mmol L^{-1} Tris-HCl), 1.5 mmol L^{-1} MgCl$_2$, 0.2 mmol L^{-1} dNTP, 0.2 μmol L^{-1} 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 1 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. Each PCR mixture (5 μL) was run on agarose gel to ensure that only the specific product was amplified. Seventeen pairs of primer sequences for amplication of exons and exon–intron boundaries of GALC gene are listed Table 2.

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

For 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 HinfI (Fig. 1a). I66M + I289V, first reported by Furuya et al. (1997), is a unique mutation identified in the Japanese population. 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′-TCATTACCTTAAA-GAGATAATCCGA-3′). The product was digested with EcoRV (Fig. 1b). 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′-ACT-AGCCACTAAATTCCAGTCGA-3′) and product was digested with SalI (Fig. 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

To screen for the 30 kb deletion mutation, a previously reported and common mutation in Caucasians, genomic DNA samples were amplified using three primers in accordance with 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). Before mutation analysis the PCR products for each exon were denatured at 94°C for 5 min, followed by gradual re-annealing at 94–25°C over 45 min to enable formation of heteroduplexes. All samples were run at three different oven temperatures, listed in Table 2. PCR-amplified products with 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 the BigDye Terminator V1.1 cycle-sequencing kit (Applied Biosystems, Warrington, UK) and the Applied Biosystems 3730 DNA analyzer.

Screening for S257F and L364R in healthy individuals

To screen for two novel missense mutations (S257F, L364R), PCR amplification of genomic DNA from 100