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Human $\alpha$-N-acetylgalactosaminidase ($\alpha$-NAGA) deficiency: new mutations and the paradox between genotype and phenotype


Abstract

Up to now eight patients with $\alpha$-NAGA deficiency have been described. This includes the newly identified patient reported here who died unexpectedly aged 1 1/2 years of hypoxia during convulsions; necropsy was not performed.

Three patients have been genotyped previously and here we report the mutations in the other five patients, including two new mutations (S160C and E193X). The newly identified patient is consanguineous with the first patients reported with $\alpha$-NAGA deficiency and neuroaxonal dystrophy and they all had the $\alpha$-NAGA genotype E325K/E325K.

Clinical heterogeneity among patients with $\alpha$-NAGA deficiency is extreme. Two affected sibs, homozygotes for E325K, are severely affected and have the signs and symptoms of infantile neuroaxonal dystrophy, but prominent vacuolisation is lacking. The mildly affected patients (two families, three patients) at the opposite end of the clinical spectrum have clear vacuolisation and angiokeratoma but no overt neurological manifestations. Two of them are homozygous for the stop mutation E193X, leading to complete loss of $\alpha$-NAGA protein. These observations are difficult to reconcile with a simple genotype-phenotype correlation and we suggest that factors or genes other than $\alpha$-NAGA contribute to the clinical heterogeneity of the eight patients with $\alpha$-NAGA deficiency.

At the metabolic level, the patients with $\alpha$-NAGA deficiency are similar. The major abnormal urinary oligosaccharides are si-alyglycopeptides of the O-glycosidic type with serine or threonine linked to the $\alpha$-N-acetylgalactosamine ($\alpha$GalNAc) moieties. Using an $\alpha$GalNAc specific lectin, intralysosomal storage products were shown in lysosomes of cultured fibroblasts from the infantile patients.

The $\alpha$-NAGA gene codes for 411 amino acids and consists of nine exons. Mutation analysis of the $\alpha$-NAGA gene showed the missense mutation E325K in the infantile cases; both patients were homozygous for this mutation. The adult patient was found to be homozygous for the missense mutation R329W.

Recently, four additional patients with $\alpha$-NAGA deficiency were reported in two independent families. The index case in the Dutch family, reported by de Jong et al., had psychomotor retardation at the age of 4 years. Screening her sibs showed that a healthy sib with $\alpha$-NAGA deficiency has no overt clinical symptoms at the age of 3 years. Chabas et al. reported adult patients of Spanish origin with a mild phenotype consisting of angiokeratoma, slight dysmorphism, lymphoedema, and prominent vacuolisation in endothelial cells, resembling the case reported by Kanzaki et al. In this paper we report the mutations of these four patients and describe a new German case with infantile $\alpha$-NAGA deficiency. The genotype and phenotype of the presently known eight cases of $\alpha$-NAGA deficiency are discussed.

Patients and methods

CASE REPORTS

The two German brothers, first reported by van Diggelen et al. and Schindler et al. with a severe infantile form of $\alpha$-NAGA deficiency, are
The mutations in all patients with α-N AG A deficiency

<table>
<thead>
<tr>
<th>Patient</th>
<th>Nucleotide change* (exon/position)</th>
<th>Protein change*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1.1</td>
<td>G11005A (8/8)</td>
<td>E325K/E325K</td>
<td>13</td>
</tr>
<tr>
<td>D1.2</td>
<td>G11005A (8/8)</td>
<td>E325K/E325K</td>
<td>13</td>
</tr>
<tr>
<td>D1.3</td>
<td>C11017T (8/8)</td>
<td>S160C/E325K</td>
<td>Present primer</td>
</tr>
<tr>
<td>NL1.1</td>
<td>G4969G (11005A) (8/8)</td>
<td>Present primer</td>
<td>Present primer</td>
</tr>
<tr>
<td>NL1.2</td>
<td>C4969G (11005A) (8/8)</td>
<td>Present primer</td>
<td>Present primer</td>
</tr>
<tr>
<td>J1.1</td>
<td>G11017T (8/8)</td>
<td>R327W/R327W</td>
<td>14</td>
</tr>
<tr>
<td>E1.1</td>
<td>G5371T (5/5)</td>
<td>E193X/E193X</td>
<td>Present primer</td>
</tr>
<tr>
<td>E1.2</td>
<td>G5371T (5/5)</td>
<td>E193X/E193X</td>
<td>Present primer</td>
</tr>
</tbody>
</table>

* Nucleotide and amino acid numbering is according to Yamauchi et al. and accession No M59199.

The mutations in the α-NAGA gene according to accession No M59199.

The newly diagnosed German patient D2.1 lived in the same village as the D1 family and is consanguineous with the German patients in family D1. The pregnancy and development up to the age of 6 months were normal. From 7 months, the patient had several convulsions, most of them during fever caused by upper respiratory tract infections. He died at the age of 18 months of hypoxia during a prolonged convulsion causing apnoea. During the last 3 months of his life, his development seemed to have stopped. He had never been admitted to a hospital and necropsy was not performed.

The two Dutch patients reported by de Jong et al. are designated NL1.1 and NL1.2. Patient NL1.1 was last seen at the age of 3-8 years and was retarded. After the age of 2-4 years she had four convulsions, mostly associated with fever, despite treatment with antiepileptic drugs. In retrospect, the retardation began after a severe episode around the age of 1 year. The patient had a high fever, uncontrollable convulsions, and attacks of apnoea and bradycardia which required admission to the intensive care unit. The fever and convulsions responded to medication, but a few days after debulking she developed pneumonia with high fever and the clinical signs of sepsis with multiple organ failure, from which she recovered slowly without convulsions. Her brother (NL1.2) appeared healthy at the age of 3 years on visual examination, but neurological tests have not been performed.

The Spanish patients reported by Chabas et al. are designated E1.1 and E1.2.

**Skin fibroblasts** were cultured according to routine procedures in Ham’s F10 medium supplemented with 10% fetal bovine serum and antibiotics. The cells were harvested with trypsin seven days after the last subculture and were stored at −70°C until use.

ξ-N-acetylgalactosaminidase (zNAGA) activity was assayed in homogenates prepared by sonication of cultured fibroblasts in water. Reaction mixtures consisted of 10 μl homogenate (30 μg protein for patient materials or 5 μg for controls) and 20 μl 1 mmol/l MUζGalNAc, Moscerdam Substrates, Rotterdam) in McIlvain’s phosphate/citrate buffer, pH 4.7. After incubation for one hour at 37°C, the reactions were terminated by the addition of 200 μl 0.5 mol/l Na3CO3/NaHCO3, pH 10.7, and the fluorescence of 4-methylumbelliferone (MU) was measured with a Fluoroskan (Titer-tek) fluorimeter. β-galactosidase activity, using an MU substrate, and protein content of cell homogenates was determined as described previously. β-galactosidase was also determined with the disaccharide Galβ1–3GalNAc (Oxford GlycoSystems) in a galactose dehydrogenase based assay. Reaction mixtures (30 μl) contained 20 μg protein from sonicated fibroblast homogenates, 2-7 mmol/l of the disaccharide, 67 mmol/l NaCl in 67 mmol/l sodium acetate buffer, pH 4.3, and were incubated for three hours at 37°C, after which 200 μl 0.1 mol/l Tris/HCl, pH 8-6 containing 0.6 mmol/l NAD+ and 0.13 U galactose dehydrogenase (Boehringer) was added. Free galactose was then determined by measuring the absorbance at 340 nm, after incubation for 25 minutes at room temperature.

Neuraminidase activity was determined by the standard method using the MU substrate as well as with a disialyltetraose substrate (DST, NeuAco2–3Galβ1–3[NeuAco2–6]GalNAc, Oxford GlycoSystems). Reaction mixtures (30 μl) contained 50 μg protein from fibroblast homogenates (Potter homogenised), 1.3 mmol/l DST, 67 mmol/l NaCl in 67 mmol/l sodium acetate buffer, pH 4.3, and were incubated for three hours at 37°C. Then free N-acetyl neuraminic acid (NANA) was purified as previously described and determined with the thiobarbituric acid method as described by Denny et al.

**IMMUNOBLOTTING**

The polyclonal antibody against human α-NAGA, raised in rabbits, was used at a 1000 fold dilution and immunoblotting was performed as previously described.

**LABELLING OF α-GALNAC MOIETIES BY LECTIN HISTOCHEMISTRY**

The lectin from Helix pomatia (Sigma), which is specific for αGalNAc, was used to stain...
panel: TaqI digests of exon 8 of the NL1 patients, their parents, and patient D2.1. Bottom panel: BsgI digests of exon 4 of NL1 patients, their parents, and a healthy sib. Figure 1: Restriction enzyme analysis of PCR amplified α-NAGA exons 8 and 4. Top panel: BsgI digests of exon 4 of NL1 patients, their parents, and a healthy sib. Bottom panel: TaqI digests of exon 8 of the NL1 patients, their parents, and patient D2.1.

Figure 2: Restriction enzyme analysis of PCR amplified α-NAGA exon 5. MseI digests of exon 5 of E1 patients and their parents.

αGalNAc containing material in cultured fibroblasts as described.

Metabolic Labelling
Cultured fibroblasts were labelled with 35S-methionine following the procedure described earlier.

DNA Amplification and Sequencing
DNA was isolated from cultured fibroblasts by standard procedures. PCR amplification and sequencing was performed according to Hermans et al. Restriction enzyme digestions (TaqI, Boehringer; BsgI and MseI, BioLabs) were performed according to the manufacturers’ instructions.

Results
The Genotype of α-NAGA Deficiency
Using the intron primer sets shown in Table 1, DNA fragments encompassing the entire open reading frame of the nine exons of the α-NAGA gene were amplified and sequenced (except for patient D2.1); this included all flanking introns (Table 1).

Since patient D2.1 is consanguineous with the D1 patients, only exon 8 was analysed, and the presence of the G11005A mutation (E325K) was confirmed (Table 2). This mutation destroys a TaqI restriction site and its presence could be confirmed by TaqI digestion of the 351 bp long PCR products of exon 8. The digestion of the normal PCR product into fragments of 174 and 177 bp was not observed in the PCR products of the mutant alleles (Fig 1). Sequence analysis of the sense and antisense strands as well as restriction enzyme analysis showed that patient D2.1 is homozygous for the E325K mutation (Fig 1). We obtained the same results for the D1 patients (data not shown).

Sequence analysis of all α-NAGA exons of the Dutch sibs (NL1) showed two different mutations. The C11017G substitution in exon 4 (S160C) could be confirmed with the restriction enzyme BsgI (the mutation creates a new restriction site). Fig 1 shows that the affected sibs are heterozygous for this mutation which is of maternal origin. The paternal mutation in these sibs is the previously described E325K mutation of the German patients. The father of the Dutch patients is not of German ancestry. The healthy sib of the NL1 patients showed the normal pattern in both exons 4 and 8, indicating that she is not a carrier of either mutation (Fig 1). The S160C mutation was not detected in 80 Dutch white control alleles.

Sequence analysis showed that both Spanish (E1) patients were homozygous for the nonsense mutation E193X. Homozygosity was confirmed using the restriction enzyme MseI (the mutation creates a new restriction site). In agreement with this observation, both parents of the E1 sibs showed a heterozygous pattern after MseI digestion of their PCR product of exon 5 (Fig 2).

The entire sequenced parts of the α-NAGA gene (Table 1) were identical in all patients and two controls (with the exception of the mutations). Differences, however, were found with the published sequence of the α-NAGA gene (Wang and Desnick, accession No M59199). In intron 1, starting at position 3516, we detected CCGTTGCCCAG (discrepancies underlined, three Gs in sequence M59199). In exon 3 we detected three polymorphisms (no amino acid changes) in all samples analysed, which had previously been published by Yamachi et al. In all the DNA samples we tested, a C was found at position 4239 instead
Table 3  α-NAGA activity in fibroblasts from patients with infantile and adult α-NAGA deficiency

<table>
<thead>
<tr>
<th></th>
<th>α-NAGA activity (nmol/h/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infantile cases</td>
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</tr>
<tr>
<td>D1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>D1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>D2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>D2.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Adult patients</td>
<td></td>
</tr>
<tr>
<td>E1.1</td>
<td>3.2</td>
</tr>
<tr>
<td>E1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Control range</td>
<td></td>
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<tr>
<td>Range</td>
<td>40-130</td>
</tr>
<tr>
<td>(mean)</td>
<td>(81)</td>
</tr>
</tbody>
</table>

of an A and at position 4263 there was a T instead of a C. In both alleles of the two controls and in one allele of the NL1 patients, an A→G substitution at position 4293 was found. In intron 3, starting at position 4348 we found four Cs instead of three Cs and starting at position 4364 we found GGCC instead of GCGC. In intron 5, at position 5404 we found a C→G substitution.

THE BIOCHEMICAL PHENOTYPE OF α-NAGA DEFICIENCY

We studied the effect of these point mutations on the steady state level of α-NAGA activity and observed differences among the various patients. The highest activity was measured in fibroblasts of patient NL1.1. About half this activity was present in cells from the D2.1 and D1 patients, whereas α-NAGA activity in the E1 patients was barely detectable (table 3).

These low residual activities and their differences are genuine since the activities are linear with the amount of protein and incubation time and reproducible (data not shown). Using a polyclonal antibody against α-NAGA, we showed that in all cases the deficiency of α-NAGA enzyme activity was associated with undetectable levels of α-NAGA protein (fig 3). Metabolic labelling of cultured fibroblasts for four hours, followed by immunoprecipitation of native α-NAGA protein showed that the synthesis of precursor α-NAGA (50 kDa) is normal in the NL1 patients and patient D1.2 (fig 4). The E1 patients, on the other hand, do not synthesise any α-NAGA protein. Maturation of newly synthesised α-NAGA was investigated after a 17 hour chase period and mature enzyme (45 kDa) was not detectable in the German patient D1.2, whereas a small amount was present in the NL1 patients (fig 4).

At the cellular level, α-NAGA deficiency was reflected in the lysosomal storage of αGalNAc containing compounds (fig 5), which were identified with the αGalNAc specific lectin from H. pomatia. On the other hand all patients with α-NAGA deficiency excrete sialylglycopeptides, the new patient D2.1 included (data not shown). Unlike the unknown structure of the intralysosomal storage products, the abnormal urinary excretion products have been fully characterised. We have investigated whether fibroblast homogenates of the most severe patient with α-NAGA deficiency (D1.2) can hydrolyse the core structures of these urinary compounds: NeuAcα2→3Galβ1→3 [NeuAcα2→6]GalNAc and Galβ1→3GalNAc. The results show that fibroblasts with α-NAGA deficiency are fully normal in their sialidase and β-galactosidase activity towards these oligosaccharides (table 4).
Deficiency of α-N-acetylgalactosaminidase (α-NAGA) would imply major urinary excretion products containing terminal αGalNAc residues at the non-reducing end; however, this is not observed. All patients with α-NAGA deficiency excrete sialylglycopeptides with terminal neuraminic acid and the α-GalNAc moiety is internal; α linked to serine or threonine.24 The structures are identical to those present in O linked glycoproteins. Using their core structure (NeuAc<sub>2</sub>—3Gal<sub>1</sub>—3∥GalNAc and Gal<sub>1</sub>—3GalNAc) we showed that fibroblasts from the severe D1.2 patient (table 5) can hydrolyse sialic acid and galactosecontaining lysosomal storage products (resynthesis) has been reported for aspartylglucosaminuria25,26 and β-mannosidosis.27 Although we have shown an αGalNAc containing lysosomal storage product in fibroblasts of all patients (we did not have permission to investigate fibroblasts from the Japanese patient), the structure of these compounds is still unknown. Studies are in progress to identify these primary lysosomal storage products.

α-NAGA deficiency is one of the rarest and probably most heterogeneous lysosomal storage disorders. At present, only eight patients are known from five families of German (D), Japanese (J), Dutch (NL), and Spanish (E) descent, including the new case reported here (table 5). Clinical variability is common in lysosomal storage disorders but overlapping signs and symptoms of subtypes is the rule. In α-NAGA deficiency, however, infantile and adult patients have no obvious overlap of signs and symptoms. This led us to consider the possibility that the phenotype of the eight patients is not solely determined by the α-NAGA deficiency. Three observations are difficult to reconcile with a simple genotype-phenotype correlation.

(1) At the histological level, prominent vacuolisation, the hallmark of lysosomal storage disorders, was observed in dermal endothelial cells of the late onset patients (patient J1.1,15 patients of the E1 family38) but was not observed in the severely affected patients of the D1 family.2 This is remarkable since vacuolisation is usually prominent in the most severe subtype of lysosomal storage disorders and less evident, or even absent, in the milder subtypes.28 The most striking histopathology in the infantile patients (D1) is non-lysosomal, the presence of "spheroids" in axons. Although axonal spheroid formation has been observed in other lysosomal storage disorders,29,30 it is a peculiar phenomenon in the absence of clear lysosomal histopathology and difficult to explain.

(2) Enzyme activity is often undetectable in the most severe subtype of a lysosomal disorder whereas patients with milder subtypes have residual activity. This has conclusively been shown for metachromatic leucodystrophy and Tay-Sachs disease31 and for Pompe's disease.32 Such a logical correlation is missing in α-NAGA deficiency. We found a stop mutation (E193X), deleting half the protein, in the mildly affected Spanish patients (E1). A translation product was not detectable by immunoblotting, metabolic labelling of newly synthesised αGalNAc did not indicate the presence of a truncated protein, and enzyme activity was virtually zero. This indicates that these patients have, in essence, null alleles and one would expect the most severe phenotype. In contrast, these patients presented with a mild, late onset disease, whereas the most severely affected patients (D1) have significant, albeit very low,
residual enzyme activity. A logical correlation was similarly lacking in studies of Wang et al.\(^3\)
comparing the severe German patients (D1) and the Japanese patient J1.1 with adult a-NAGA
deficiency.\(^4\) The authors could not detect significant a-NAGA enzyme activity in either
case.

(3) After the clinical description of the first patients with a-NAGA deficiency (D1), many
laboratories, including ours, have screened Scitlberger patients (infantile neuroaxonal dys­
trophy) for a-NAGA deficiency, but a second patient with combined a-NAGA deficiency and
neuroaxonal dystrophy has not been found.

These three observations indicate that factors other than a-NAGA contribute to the pheno­
typic variation of the patients. The simplest explanation would be that the severe infantile
patients (D1) have a "double disease", neuroaxonal dystrophy in addition to a-NAGA deficiency,
without a causal relationship. Accidental occurrence of two independent mono­
genic diseases in one patient can be observed, particularly in consanguineous families (the
parents of the patients D1.1 and D1.2 are consanguineous). For example, in one of the
contributing laboratories (Rotterdam) four cases are known: glycogenosis II combined with
methylnalactic aciduria, neurofibromatosis I with multiple sulphatase deficiency, Niemann–
Pick type C with Hurler disease, and combined Zellweger syndrome and sulphite oxidase de­
ficiency. One of the first patients with \(\beta\)-mannosidosis\(^5\) had mucopolysaccharidosis type III
A as a second disease.\(^3^4\)

If the severely affected German patients (D1) did have a double disease, this phenotype is
not representative of "true" a-NAGA deficiency. The phenotype associated with complete
a-NAGA deficiency would be a mild, late onset disease with angiokeratoma manifested in
patient J1.1 and the Spanish patients (E1).

The Dutch patient NL1.2, aged 3-3 years without overt signs or symptoms, could be a pre­
clinical case of a-NAGA deficiency detected through screening. The newly identified mis­
sense mutation in the Dutch patients (S160C) results in 4% residual activity, the highest level
among all the patients with a-NAGA deficiency; this may contribute to the mild pheno­
type. Only in these patients a small amount of mature a-NAGA protein was found 17 hours
after metabolic labelling of the enzyme in cultured fibroblasts, which is in agreement with
the significant residual enzyme activity. The newly identified patient D2.1, who had identical
a-NAGA mutations as the patient with neuroaxonal dystrophy (D1), could in time have
provided important information as to the relation of a-NAGA deficiency and neuro­
axonal dystrophy, but the patient died at the age of 1\(\frac{1}{2}\) years of hypoxia during a convulsion
without being examined histologically and neurologically.

In summary, all the genetic, biochemical, histological, and clinical data of all patients with
a-NAGA deficiency strongly suggest that a-NAGA deficiency is not a single disease entity.
The possibility that factors other than a-NAGA play a major role in determining the phenotypic
variation of the eight known patients with a-NAGA deficiency deserves serious con­
sideration. The future discovery of new patients with a-NAGA deficiency will eventually estab­
lish which phenotypes are solely caused by a-NAGA deficiency and if other factors/genes play
a role in other phenotypes. This will probably take a long time since the discovery of the first
eight patients took almost a decade. It will there­
fore pay to study the association of a-NAGA deficiency and neuroaxonal dystrophy in an a-
NAGA knock-out mouse.

We thank Professor Hans Gajardo for his continuing support
and Torn de Vries Lemisch for photography. Dr Aikiko Tsuji
is gratefully acknowledged for preparing the antiserum against
a-NAGA. The authors thank Dr E Rodriguez-Diaz (Hospital
Valle del Nalon, Asturias) and Dr M Aparicio (Hospital Clinico
Universitario, Salamanca) for referring the Spanish patients E1.
We thank Dr Steven U Wolkley (New York) for stimulating
discussions about the relationship between neuroaxonal spher­
oids and lysosomal storage.

### Table 5: Summary of all patients with a-NAGA deficiency

| Age | Neurological signs | Physical signs | Histology | Neuroaxonal dystrophy | a-GalNAc lectin staining | Neutrophil 
|---|---|---|---|---|---|---|
| Age at onset | Convulsions during fever | Dysmorphism | Neutrophilic dysmorphism | a-GalNAc lectin staining | Neutrophil 
| Present age (age at death) | Epilepsy | Organomegaly | Angiokeratoma | | |
| 1 y | + | + | + | + | + | + |
| 6 mth | + | + | + | + | + | + |
| 7 mth | + | + | + | + | + | + |
| 1 y | + | + | + | + | + | + |
| 3-6 y | + | + | + | + | + | + |
| No symptoms? | + | + | + | + | + | + |

**Mutations**

- Homozygous E325K
- S160C/E325K

**Adult a-NAGA deficiency**

<table>
<thead>
<tr>
<th>J1.1</th>
<th>E1.1</th>
<th>E1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Ni</td>
<td>Ni</td>
</tr>
<tr>
<td>28 y</td>
<td>14 y</td>
<td>38 y</td>
</tr>
<tr>
<td>1 y</td>
<td>42 y</td>
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</tr>
<tr>
<td>51 y</td>
<td>2 y</td>
<td></td>
</tr>
<tr>
<td>3 y</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**

- \(\ast\) D1: van Diggelen et al.\(^3\) Schindler et al.\(^2\)
- \(\dagger\) D2.1: present paper.
- \(\ddagger\) NL1.1: de Jong et al.\(^3\)
- \(\text{J1.1: Kanski et al.}^3\)
- \(\text{E1.1: Chabas et al.}^3\)
- \(\text{NI = not investigated.}^3\)
- \(\text{NA** = material not available.}^3\)
- \(\text{†† Wang et al.}^3\)

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*We thank Professor Hans Gajardo for his continuing support and Torn de Vries Lemisch for photography. Dr Aikiko Tsuji is gratefully acknowledged for preparing the antiserum against a-NAGA. The authors thank Dr E Rodriguez-Diaz (Hospital Valle del Nalon, Asturias) and Dr M Aparicio (Hospital Clinico Universitario, Salamanca) for referring the Spanish patients E1. We thank Dr Steven U Wolkley (New York) for stimulating discussions about the relationship between neuroaxonal spheroids and lysosomal storage.*


23 Hermanns MMP, Kros MA, de Graaff E, Oostra BA, Reuser AJJ. Two mutations affecting the transport and maturation of lysosomal α-glucosidase in an adult case of glycosogenosis type II. _Hum Mutat_ 1992;2:268-73.


