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


Abstract
Up to now eight patients with α-NAGA deficiency have been described. This includes the newly identified patient reported here who died unexpectedly aged 1½ 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 α-NAGA deficiency and neuroaxonal dystrophy and they all had the α-NAGA genotype E325K/E325K.

Clinical heterogeneity among patients with α-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 α-NAGA protein. These observations are difficult to reconcile with a simple genotype-phenotype correlation and we suggest that factors or genes other than α-NAGA contribute to the clinical heterogeneity of the eight patients with α-NAGA deficiency.

At the metabolic level, the patients with α-NAGA deficiency are similar. The major abnormal urinary oligosaccharides are sialylglycopeptides of the O linked type. Our enzymatic studies indicate that these compounds are not the primary lysosomal storage products.

Keywords: α-N-acetylgalactosaminidase; α-NAGA deficiency; processing.

In 1987 two German infants were reported by Van Diggelen et al with a profound deficiency of the lysosomal enzyme α-N-acetylgalactosaminidase (α-NAGA). Schindler et al reported that the patients had infantile neuroaxonal dystrophy without visceral involvement or dysmorphism. Two years later Kanzaki et al reported the second independent case of α-NAGA deficiency with an entirely different clinical phenotype. This patient had a late onset disease with slight facial coarseness, disseminated angiokeratoma, and mild intellectual impairment (IQ=70), but without neurological symptoms. Unlike the infantile cases, this patient had prominent vacuolisation in all dermal cells, most prominently in vascular and lymphatic endothelial cells and eccrine sweat gland cells, but also in dermal neural cells and fibroblasts. The glomerular endothelial cells but not the epithelial kidney cells are involved and also blood lymphocytes are vacuolised.

These three patients shared, however, the abnormal urinary excretion of specific oligosaccharides. The major compounds are sialylglycopeptides of the O-glycosidic type with serine or threonine linked to the α-N-acetylgalactosamine (αGalNAc) moieties. Using an αGalNAc specific lectin, intralysosomal storage products were shown in lysosomes of cultured fibroblasts from the infantile patients.

The α-NAGA gene codes for 411 amino acids and consists of nine exons. Mutation analysis of the α-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 α-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 α-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 α-NAGA deficiency. The genotype and phenotype of the presently known eight cases of α-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 α-NAGA deficiency, are
Table 1. Oligonucleotides used to sequence and PCR amplify the coding regions of the α-NAGA gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequences (location in gene)</th>
<th>Length of fragment sequenced (relative position to exon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>s: GCCTAGGTTGAGGGGG (1646-1663)</td>
<td>228 bp (−60 to +51)</td>
</tr>
<tr>
<td>2</td>
<td>s: AGCTGGGGGGGCACTGGA (3479-3498)</td>
<td>233 bp (−11 to +2)</td>
</tr>
<tr>
<td>3</td>
<td>s: GCACAGGTGTGGGGCTGA (3606-3701)</td>
<td>308 bp (−29 to +32)</td>
</tr>
<tr>
<td>4</td>
<td>s: GCCCTAACAGGATTGATGGTTG (3839-4408)</td>
<td>260 bp (−17 to +23)</td>
</tr>
<tr>
<td>5</td>
<td>s: GTCTCCTGCTGACCCCTGCTOT (4777-4796)</td>
<td>204 bp (−22 to +29)</td>
</tr>
<tr>
<td>6</td>
<td>s: CTTGCTGCTGCTGCTCCTCCTG (5823-5850)</td>
<td>248 bp (−7 to +18)</td>
</tr>
<tr>
<td>7</td>
<td>s: AGAGGAGGGGCCCTGATGG (5833-5850)</td>
<td>282 bp (−4 to +13)</td>
</tr>
<tr>
<td>8</td>
<td>s: CTCACGGGCCTGCTGCTG (6125-6148)</td>
<td>226 bp (−12 to +9)</td>
</tr>
</tbody>
</table>

* Exon number and position of nucleotide in α-NAGA gene according to accession No M59199.

** Position upstream of start codon in exon 1.
*** Position downstream of stop codon in exon 9.

Table 2. The mutations in all patients with α-NAGA deficiency

<table>
<thead>
<tr>
<th>Patient</th>
<th>Nucleotide change* (exon/locus)</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>G11005A (8/8)</td>
<td>E325K/E325K</td>
<td>Present</td>
</tr>
<tr>
<td>NL1.1</td>
<td>C4969G/G11005A (4/8)</td>
<td>S160C/E325K</td>
<td>Present</td>
</tr>
<tr>
<td>NL1.2</td>
<td>C4969G/G11005A (4/8)</td>
<td>S160C/E325K</td>
<td>Present</td>
</tr>
<tr>
<td>J1.1</td>
<td>C11017T (8/8)</td>
<td>E3127R/E325K</td>
<td>14</td>
</tr>
<tr>
<td>E1.1</td>
<td>G357T1T (5/5)</td>
<td>E3127R/E325K</td>
<td>Present</td>
</tr>
<tr>
<td>E1.2</td>
<td>G357T1T (5/5)</td>
<td>E3127R/E325K</td>
<td>Present</td>
</tr>
</tbody>
</table>

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

The exons in which the mutation was found are shown in brackets.
Keulemans et al. D2-1 E1-2

Panel: TaqI digests of exon 8 of the N L J patients, their parents and patient D2.1.

Figure 1 Restriction enzyme analysis of PCR amplified α-NAGA exons 8 and 4. Top panel: BsgI digests of exon 4 of E1 patients and their parents. Bottom panel: TaqI digests of exon 8 of the N L J patients, their parents, and patient D2.1.

DNA was isolated from cultured fibroblasts by methionine following the procedure described earlier. PCR amplification and restriction enzyme analysis of their PCR products of the mutant alleles (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 Msel (the mutation creates a new restriction site). In agreement with this observation, both parents of the E1 sibs showed a heterozygous pattern after Msel 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 CCGTTGCCCCC (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 of a G.}

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 Msel, 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 intron regions (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 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.

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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 \(\alpha\)-NAGA DEFICIENCY

We studied the effect of these point mutations on the steady state level of \(\alpha\)-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 \(\alpha\)-NAGA activity in the E1 patients was barely detectable (table 3).

The low residual activities and their differences are genuine since the activities are linear with the amount of protein and incubation time and reproduceable (data not shown). Using a polyclonal antibody against \(\alpha\)-NAGA, we showed that in all cases the deficiency of \(\alpha\)-NAGA enzyme activity was associated with undetectable levels of \(\alpha\)-NAGA protein (fig 3). Metabolic labelling of cultured fibroblasts for four hours, followed by immunoprecipitation of native \(\alpha\)-NAGA protein showed that the synthesis of precursor \(\alpha\)-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 \(\alpha\)-NAGA protein. Maturation of newly synthesised \(\alpha\)-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, \(\alpha\)-NAGA deficiency was reflected in the lysosomal storage of \(\alpha\)GalNAc containing compounds (fig 5), which were identified with the \(\alpha\)GalNAc specific lectin from \(H\) pomatia. On the other hand all patients with \(\alpha\)-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 \(\alpha\)-NAGA deficiency (D1.2) can hydrolyse the core structures of these urinary compounds: NeuAc2→3Gal/β1→3 [NeuAc2→6]GalNAc and Gal/β1→3GalNAc. The results show that fibroblasts with \(\alpha\)-NAGA deficiency are fully normal in their sialidase and \(\beta\)-galactosidase activity towards these oligosaccharides (table 4).
**Figure 5** Intracellular labelling of α-N-acetylgalactosamine containing compounds in fibroblasts from patients with α-NAGA deficiency. Lectin histochemistry, using the αGalNAc specific lectin from Helix pomatia, was carried out on cultured fibroblasts from a control (A), the patients D1.2 (B), D2.1 (C), NL1.1 (D), E1.1 (E), and E1.2 (F).

**Table 4** Degradation of core structures of O-glycosidic glycoproteins by neuraminidase and β-galactosidase.

<table>
<thead>
<tr>
<th>Nonaminidase activity*</th>
<th>β-galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MU-NANA†</td>
<td>DSt‡</td>
</tr>
<tr>
<td>α-NAGA deficiency (D1.2)</td>
<td>78</td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
</tr>
<tr>
<td>GM2-gangliosidosis</td>
<td>35</td>
</tr>
<tr>
<td>Sialidosis</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Enzyme activities: nmol/h/mg.
† 4-methylumbelliferyl-α-D-N-acetylneuraminic acid.
§ 4-methylumbelliferyl-β-D-galactoside.

**Discussion**

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. The structures are identical to those present in O linked glycoproteins. Using their core structure (NeuAcα2→3Galβ1→3[NeuAcα2→6]GalNAc and Galβ1→3GalNAc) we showed that fibroblasts from the severe D1.2 patient (table 5) can hydrolyse sialic acid and galactose from these compounds. This indicates that the sialylglycopeptides from urine are not the primary lysosomal storage products, which will probably be GalNAc1→Ser/Thr. Minor amounts of these compounds have been detected in urine of the D1 patients. These putative primary storage products could subsequently serve as acceptor for the synthesis of the O linked type oligosaccharides. Addition of glycosides to primary lysosomal storage products (resynthesis) has been reported for aspartylglucosaminuria and β-mannosidosis. 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, patients of the EI family) but was not observed in the severely affected patients of the D1 family. 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. 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, 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 disease and for Pompe's disease. 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 (EI). A translation product where the peculiar phenomenon in the absence of clear lysosomal histopathology and difficult to explain.

(3) Axonal spheroid formation has been observed in other lysosomal storage disorders, such as metachromatic leucodystrophy, Tay-Sachs disease, and Pompe's disease. Although axonal spheroid formation has been observed in other lysosomal storage disorders, such as metachromatic leucodystrophy, Tay-Sachs disease, and Pompe's disease, it is a peculiar phenomenon in the absence of clear lysosomal histopathology and difficult to explain.
residual enzyme activity. A logical correlation was similarly lacking in studies of Wang et al., comparing the severe German patients (D1) and the Japanese patient J1.1 with adult α-NAGA deficiency. The authors could not detect significant α-NAGA enzyme activity in either case.

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

These three observations indicate that factors other than α-NAGA contribute to the phenotypic variation of the patients. The simplest explanation would be that the severe infantile patients (D1) have a "double disease", neuroaxonal dystrophy in addition to α-NAGA deficiency, without a causal relationship. Accidental occurrence of two independent monogenic 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 methylmalonic aciduria, neurofibromatosis I with multiple sulphatase deficiency, Niemann-Pick type C with Hurler disease, and combined Zellweger syndrome and sulphite oxidase deficiency. One of the first patients with β-mannosidosis had mucopolysaccharidosis type III A as a second disease.

If the severely affected German patients (D1) did have a double disease, this phenotype is not representative of "true" α-NAGA deficiency. The phenotype associated with complete α-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 preclinical case of α-NAGA deficiency detected through screening. The newly identified missense mutation in the Dutch patients (S160C) results in 4% residual activity, the highest level among all the patients with α-NAGA deficiency; this may contribute to the mild phenotype. Only in these patients a small amount of mature α-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 α-NAGA mutations as the patients with neuroaxonal dystrophy (D1), could in time have provided important information as to the relation of α-NAGA deficiency and neuro-axonal dystrophy, but the patient died at the age of 1 ½ 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 α-NAGA deficiency strongly suggest that α-NAGA deficiency is not a single disease entity. The possibility that factors other than α-NAGA play a major role in determining the phenotypic variation of the eight known patients with α-NAGA deficiency deserves serious consideration. The future discovery of new patients with α-NAGA deficiency will eventually establish which phenotypes are solely caused by α-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 therefore pay to study the association of α-NAGA deficiency and neuroaxonal dystrophy in an α-NAGA knock-out mouse.

We thank Professor Hans Galjaard for his continuing support and Tom de Vries Lemisch for photography. Dr Akihiko Tsuji is gratefully acknowledged for preparing the antisera against α-NAGA. The authors thank Dr E Rodriguez-Diaz (Hospital Valle del Nalon, Asturias) and Dr M Aparicio (Hospital Clínico Universitario, Salamanca) for referring the Spanish patients E1. We thank Dr Steven U Walkley (New York) for stimulating discussions about the relationship between neuroaxonal spheroids and lysosomal storage.


