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Research Article

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Niemann-Pick Type B Disease

Identification of a Single Codon Deletion in the Acid Sphingomyelinase Gene and Genotype/Phenotype Correlations in Type A and B Patients

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Abstract

Types A and B Niemann-Pick disease both result from the deficient activity of the lysosomal hydrolase, acid sphingomyelinase (E.C. 3.1.4.12). Type A Niemann-Pick disease is a severe neurodegenerative disorder of infancy which leads to death by three years of age, whereas Type B disease has a later age at onset, little or no neurologic involvement, and most patients survive into adulthood. To investigate the molecular basis for the remarkable phenotypic heterogeneity, the nature of the mutations causing Type B Niemann-Pick disease in Ashkenazi Jewish patients was determined. The entire acid sphingomyelinase coding region from an Ashkenazi Jewish Type B patient was polymerase chain reaction–amplified, subcloned, and completely sequenced. A three-base deletion was identified of nucleotides 1821–1823 in the cDNA which predicted the removal of an arginine residue from position 608 of the acid sphingomyelinase polypeptide (ΔR608). The other cDNA clones from this patient had the R496L mutation previously identified in Type A Niemann-Pick disease patients. Both Ashkenazi Jewish Type B patients were heteroallelic for the ΔR608 mutation, whereas this allele was not present in 15 unrelated non-Jewish Type B patients, with the notable exception of one mildly affected patient of Arabic descent who was homoallelic for the ΔR608 mutation. These results indicate that the ΔR608 mutation predicts the Type B Niemann-Pick disease phenotype, even in the presence of the R496L Type A allele, thereby providing the first genotype/phenotype correlation for this lysosomal storage disease. Although only two patients have been studied, it appears that the ΔR608 mutation occurs frequently in Type B Niemann-Pick disease patients of Ashkenazi Jewish descent. (*J. Clin. Invest.* 1991. 88:806–810.) Key words: lysosomal storage disease • heteroallelism • genotype/phenotype correlations • polymerase chain reaction

Introduction

Niemann-Pick disease, an inborn error of sphingomyelin catabolism, results from the deficient activity of the lysosomal hydrolase, acid sphingomyelinase (sphingomyelin phosphodiesterase; E.C.3.1.4.12) (1, 2). Two allelic forms of this autosomal recessive disorder have been delineated based on their distinct phenotypes (2–6). Type A Niemann-Pick disease is a severe neurodegenerative disorder of infancy characterized by progressive psychomotor retardation, hepatosplenomegaly, and death by three years of age. In contrast, Type B Niemann-Pick disease is a nonneuronopathic disorder characterized by hepatosplenomegaly, respiratory involvement, and survival into adulthood. In addition, a third form of Niemann-Pick disease, Type C, has been described (5). However, since most of the patients with Type C Niemann-Pick disease have a defect in cholesterol esterification, it is unlikely that this form of the disease results from primary lesions in the acid sphingomyelinase gene. The clinical course of patients with Type A disease is quite uniform, whereas the severity among Type B patients is more variable. In fact, some mildly affected Type B patients may survive into the sixth decade of life (5). Both types occur more frequently among individuals of Ashkenazi Jewish descent (~ 1/40,000) than in the general population (7); however, mass screening for carrier detection in this population has been precluded due to the marked overlap of acid sphingomyelinase activities in leukocytes from obligate heterozygotes and normal individuals.

To date, biochemical investigations into the genetic heterogeneity underlying the neuronopathic Type A and nonneuronopathic Type B phenotypes have been limited since acid sphingomyelinase is difficult to purify, the commonly used enzymatic assays are insensitive and labor intensive, and monospecific antibodies have been difficult to produce (8–10). In general, Type A Niemann-Pick disease patients tend to have slightly lower residual activities in crude cell and tissue homogenates than Type B patients; however, the residual activities have not been well characterized (2, 5, 11, 12). Similarly, all of the Type A and B Niemann-Pick disease patients studied to date have had cross-reactive immunologic material in crude cell homogenates; however, accurate estimates of the amount of enzyme protein have not been determined (13, 14).

In contrast, investigations into the molecular nature of the genetic heterogeneity in Niemann-Pick disease have been facilitated by the recent isolation and characterization of the full-length cDNA encoding human acid sphingomyelinase (15, 16). The 2347-bp cDNA contained an open reading frame of 1890-bp which encoded a 629-residue polypeptide containing six potential *N*-glycosylation sites. The availability of the cDNA permitted the localization of the acid sphingomyelinase gene to the chromosomal region 11p15.1–15.4 by the use of somatic cell hybrids and in situ hybridization techniques (17). Studies also have been undertaken to determine the nature of the genetic lesions causing Type A Niemann-Pick disease. By sequencing polymerase chain reaction (PCR)[1]-amplified acid

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1. *Abbreviations used in this paper:* ASO, allele-specific oligonucleotide; PCR, polymerase chain reaction; PSA_{12}-sphingomyelin, [*N*-12(1-pyrenesulfonoyl)amido dodecanoyl] sphingomyelin.

sphingomyelinase cDNAs from a Type A patient of Ashkenazi Jewish descent, a single, exonic G to T transversion was identified which predicted an arginine to leucine substitution in position 496 of the acid sphingomyelinase polypeptide (designated R496L) (18). The R496L mutation was found in about one-third of the Ashkenazi Jewish Type A Niemann-Pick disease alleles studied; in contrast, only about 6.0% of the alleles from non-Jewish Type A patients had this mutation. Some Type A patients were homoallelic for the R496L mutation, while others were heteroallelic, indicating the occurrence of other acid sphingomyelinase mutations that caused the Type A phenotype. Interestingly, the R496L mutation also was detected in one of the two Ashkenazi Jewish Type B patients studied. Thus, efforts were undertaken to determine the molecular lesion in the other acid sphingomyelinase allele of the Ashkenazi Jewish Type B patient who was heteroallelic for the R496L mutation.

In this communication, we report the identification of a three-base deletion in the acid sphingomyelinase coding region from this Type B Niemann-Pick disease patient (proband 2) that results in the deletion of codon 608, which encoded an arginine residue (designated ΔR608). Notably, both of the unrelated Ashkenazi Jewish Type B patients analyzed were heteroallelic for the ΔR608 mutation and one of the 15 unrelated non-Jewish Type B patients studied was homoallelic. The absence of the ΔR608 mutation in 67 Type A Niemann-Pick disease alleles suggested that the ΔR608 lesion resulted in the Type B phenotype, even when heteroallelic with the R496L Type A mutation, thus providing the first genotype/phenotype correlation for Niemann-Pick disease.

Methods

Cell lines. Primary cultures of fibroblasts and lymphoblasts were established from skin biopsies and peripheral blood samples obtained with informed consent from Niemann-Pick disease patients and family members, and from normal individuals. Niemann-Pick disease lines GM00112A, GM00165, GM00370, GM00406, GM00559, GM02895, and GM03252 were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ. Cell lines 444X.F01, 534R.F03, 556X.F01, 888V.F01, 2789X.F01, 4293Q.E02, 4774Z.F01, 5113C.L01, 5115-E.F01, and 6791M.F01 were obtained from the Service de Biochimie, Hospice de Lyon, Lyon, France. Cell lines DMN 83.126, DMN 84.135, DMN 84.87, DMN 86.49, DMN 87.71, DMN 87.99, DMN 88.12, DMN 88.9, and RNS were provided by Dr. Peter Penchev, Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke. The cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% penicillin, and 1 mg/ml streptomycin by standard procedures (18). The diagnosis of Types A and B Niemann-Pick disease was based on clinical criteria (e.g., age at onset, presence of neurologic involvement, etc.) and by demonstration of markedly deficient acid sphingomyelinase activity in cultured cells (8). Clinical data on probands 1 and 2 have been published (3, 19), and information on proband 3 was provided by Dr. M. Vanier, Department of Biochemistry, Faculte de Medecine, Lyon, France.

Enzyme and protein assays. Acid sphingomyelinase activity was determined in cultured fibroblasts obtained from Niemann-Pick disease patients and normal individuals using the fluorescent natural substrate, [*N*-12(1-pyrenesulfonyl)amido dodecanoyl] sphingomyelin (PSA_{12}-sphingomyelin) as previously described (8). 1 U of activity equals that amount of enzyme that hydrolyzes 1 nmol of substrate per h. Protein determinations were performed by a modified fluorescamine assay (20).

cDNA and genomic amplification and sequencing. For acid sphingomyelinase cDNA amplification and sequencing, total RNA was isolated from cultured cells by standard procedures (21). First-strand cDNA was synthesized with reverse transcriptase from ~ 5 μg of total RNA using a cDNA synthesis kit according to the manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). The acid sphingomyelinase cDNA (~ 10% of the total reaction) was PCR-amplified (22) with *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT) using the previously described oligonucleotide primers (18). PCR was performed for 30 cycles, each consisting of denaturation for 1 min at 94°C and annealing and extension for 4 min at 72°C. After amplification, the PCR products were isolated from agarose gels and subcloned into either Bluescript KS (+) (Stratagene Inc., La Jolla, CA) or pGEM 9Zf (−) (Promega Corp., Madison, WI) vectors. For each amplified product, from 4 to 10 independent subclones were sequenced in both orientations by the dideoxy method (23).

To confirm the candidate mutation, genomic DNA was isolated from the proband (21) and a 567-bp genomic fragment containing the mutation was PCR-amplified using sense (5′-AGTAGTCGACATGGGCAGGATGTGTGG-3′) and antisense (5′-AGTAGTGTCGACTTGCCTGGTTGAACCACAGC-3′) primers synthesized on a DNA Synthesizer (model 380B; Applied Biosystems Inc., Foster City, CA) (24). The amplified genomic fragment was isolated, subcloned, and sequenced as described above for the PCR-amplified acid sphingomyelinase cDNAs.

Dot-blot analysis of the mutant alleles. For detection of the ΔR608 mutation in other Niemann-Pick disease patients, their parents and relatives, as well as normal individuals, total genomic DNA was isolated, PCR-amplified as described above, and the 567-bp acid sphingomyelinase product was analyzed by dot-blot hybridization using Zetabind nylon membranes (AMF-Cuno, Meriden, CT) and a dot-blot apparatus (Bio-Rad Laboratories, Richmond, CA). Hybridizations of the PCR product with the normal allele-specific oligonucleotide (ASO) (5′-CTCTGTGCCGCCACCTG-3′) or with the ΔR608 ASO (5′-GCTCTGTGCCACCTGAT-3′) were performed for at least 3 h at 39°C. 5′ end-labeling of the ASOs with T4 polynucleotide kinase and [γ-^{32}P]ATP (> 5,000 Ci/mmol) was performed by standard procedures (21). After hybridization, the blots were washed at room temperature for 15 min in 6× SSC containing 0.1% SDS, and then for 2 h in the same solution at either 54°C for the normal ASO or 50°C for the ΔR608 ASO. Dot-blot analysis of the R496L mutation was performed as previously described (19).

Results

Identification of the ΔR608 mutation in an Ashkenazi Jewish patient with Type B Niemann-Pick disease. Previous studies of an Ashkenazi Jewish Type B Niemann-Pick disease patient (proband 2; cell line MS 1271) indicated that he had ~ 5–8% residual acid sphingomyelinase activity and that he was heteroallelic for the R496L mutation (19). To identify the molecular lesion in his other acid sphingomyelinase allele, total RNA from the proband was reverse-transcribed, the acid sphingomyelinase coding region was PCR-amplified, and the PCR products were subcloned into plasmid vectors for DNA sequencing. A three-base deletion (CCG) of nucleotides 1821–1823 in the full-length acid sphingomyelinase cDNA (16) was identified which predicted the removal of a single arginine residue at position 608 of the acid sphingomyelinase polypeptide (designated ΔR608). The authenticity of this deletion was determined by genomic sequencing (Fig. 1) and by dot-blot hybridization of PCR-amplified genomic DNA from proband 2 and other family members using an ASO specific for the ΔR608 mutation (Fig. 2). In addition, dot-blot hybridizations confirmed the ΔR608/R496L genotype of proband 2, and dem-

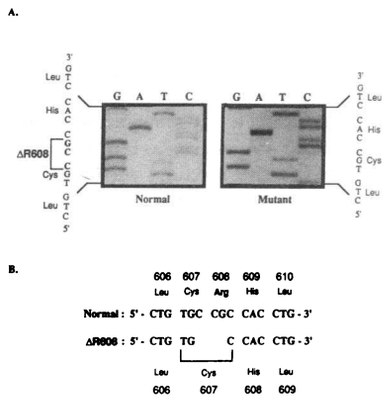

Figure 1. Identification of a three-base deletion in the acid sphingomyelinase genomic DNA from proband 2. (*A*) The methods for PCR amplification of the acid sphingomyelinase genomic DNA from proband 2, subcloning of the PCR products, and DNA sequencing are described in the text. A small area of the genomic sequence obtained from a normal individual (*left*) and proband 2 (*right*) is shown. (*B*) A schematic representation of the ΔR608 mutation.

onstrated that the ΔR608 and R496L mutations were transmitted from his father and mother, respectively. Proband 2's brother did not receive either mutant acid sphingomyelinase allele. The ΔR608 mutation was not identified in over 100 acid sphingomyelinase alleles from normal individuals, indicating that the deletion was not a polymorphism.

Occurrence of the ΔR608 mutation in types A and B Niemann-Pick disease. Table I shows the occurrence of the ΔR608 mutation in the acid sphingomyelinase alleles of other patients and obligate heterozygotes with Types A and B Niemann-Pick disease, as determined by dot-blot analysis of PCR-amplified genomic DNA. Interestingly, a second, unrelated Ashkenazi Jewish Type B Niemann-Pick disease patient was heteroallelic for the ΔR608 mutation and another, unknown mutant acid sphingomyelinase allele. Of the 15 non-Jewish Type B patients studied only one, an Arab from Algeria (proband 3; cell line 534R.F03), was homoallelic for this mutation. This 21-yr old male has a mild Type B phenotype (Vanier, M. T., personal communication). Of the 67 acid sphingomyelinase alleles from Type A Niemann-Pick disease patients or obligate heterozygotes (26 and 19 unrelated Ashkenazi Jewish and non-Jewish individuals, respectively), none had the ΔR608 mutation.

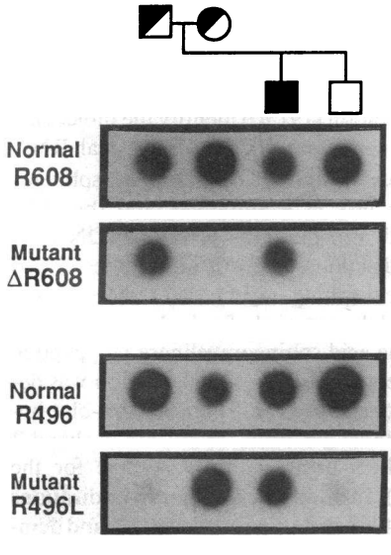

Figure 2. Genotype analysis of proband 2 and family members by dot-blot hybridization. The conditions used for dot-blot hybridization of PCR-amplified genomic DNA with the R496L and ΔR608 ASOs are described in the text.

Table I. Frequency of the ΔR608 Mutation in Ashkenazi Jewish and Non-Jewish Families with Types A and B Niemann-Pick Disease

Source	Unrelated families studied	Mutant alleles studied	% ΔR608
Type B disease			
Ashkenazi Jewish	2	4	50
Non-Jewish	15	30	6.7
Type A disease			
Ashkenazi Jewish	17	31	0.0
Non-Jewish	18	36	0.0

Comparison of the residual acid sphingomyelinase activities in Type A and B Niemann-Pick disease patients. Table II shows the acid sphingomyelinase activities in cultured fibroblasts from probands 1, 2, and 3, which were determined using the fluorescent natural substrate, PSA_{12}-sphingomyelin. Normal individuals had a mean activity of 46.3 nmol cleaved/h per mg. In contrast, Type A proband 1, who was homoallelic for R496L, had < 1% of normal activity. Type B proband 2, whose genotype was R496L/ΔR608, had a residual activity of ~ 5% of normal, whereas proband 3 who was homoallelic for ΔR608 had ~ 13% of normal mean acid sphingomyelinase activity, indicating that the ΔR608 allele expressed functional acid sphingomyelinase activity in a dosage-dependent manner.

Discussion

In 1966, Brady et al. reported that the primary enzymatic defect in Type A Niemann-Pick disease was the deficient activity of acid sphingomyelinase (1). In the following year, Schneider and Kennedy demonstrated that acid sphingomyelinase activity also was markedly decreased in patients with the milder, visceral form of Niemann-Pick disease now known as Type B disease (2). Subsequent biochemical analyses of additional patients confirmed these findings (10–12) and somatic cell genetic studies demonstrated that the mutations causing Types A and B disease were allelic (6). These findings stimulated investigators to speculate that the remarkable clinical heterogeneity observed among Type A and B Niemann-Pick disease patients

Table II. Genotype/Phenotype Correlations in Niemann-Pick Types A and B Disease

Genotype	Phenotype	Acid sphingomyelinase activity: mean (range)	Acid sphingomyelinase activity: % of normal mean
		nmol/h per mg	
R496L/R496L (proband 1)	Type A	0.33 (0.21–0.47)	0.7
R496L/ΔR608 (proband 2)	Type B	2.23 (1.8–2.3)	4.8
ΔR608/ΔR608 (proband 3)	Type B	5.95 (5.1–6.9)	12.8

The mean acid sphingomyelinase activity in three normal individuals was 46.3 nmol/h per mg (range 37.5–61.0 nmol/h per mg).

was due to different mutations in the acid sphingomyelinase gene which resulted in altered enzyme polypeptides that expressed varying amounts of residual activity (e.g., 13, 14). However, efforts to reliably predict either the disease subtype or the severity of Type B patients by the amount of residual acid sphingomyelinase activity have not been possible, in part due to the inability of assay procedures to accurately distinguish between patients with low levels of residual acid sphingomyelinase activity and/or the presence of the neutral sphingomyelinase activity in cell homogenates (25). In addition, the inability to reliably discriminate obligate heterozygotes for Types A and B Niemann-Pick disease from noncarriers by the determination of acid sphingomyelinase activity in isolated leukocytes has precluded carrier screening for Niemann-Pick disease.

The recent cloning and sequencing of the acid sphingomyelinase cDNA (15, 16) has permitted identification of the first mutations which result in Types A and B Niemann-Pick disease. Previously, the R496L mutation, due to a single G to T transversion, was found in 32% of the Ashkenazi Jewish Type A Niemann-Pick disease alleles studied. In contrast, it was present in only 5.6% of the non-Jewish Type A alleles analyzed (19). In addition, proband 2, an Ashkenazi Jewish Type B Niemann-Pick disease patient was found to be heteroallelic for the R496L mutation. In our study, a three-base deletion (ΔR608) in the acid sphingomyelinase gene was identified as the other mutation in proband 2. As shown in Fig. 1 *B*, the three-base deletion, CCG, removed the last nucleotide of codon Cys-607 (TGC) and the first two nucleotides of codon Arg-608 (*CGC*). The new codon 607 formed by this deletion, TGC, also encoded a cysteine residue. Thus, the deletion resulted in the elimination of a single codon, 608, which encoded an arginine residue.

Detection of the R496L and ΔR608 mutations in patients with Types A and B Niemann-Pick disease has permitted genotype/phenotype correlations and provided insights into the function of the altered enzymes encoded by these mutant alleles (Table II). The homoallelic (R496L/R496L) Type A patient who expresses < 1% of normal acid sphingomyelinase activity in cultured fibroblasts indicates that the R496L mutation encodes an acid sphingomyelinase polypeptide with little, if any, catalytic activity and/or markedly decreased stability, thereby resulting in the neuronopathic phenotype. In contrast, both heteroallelic (R496L/ΔR608) and homoallelic (ΔR608/ΔR608) Type B patients express an enzyme with sufficient residual activity to prevent neurologic manifestations. These findings suggest that the substitution of an arginine for a leucine at position 496 was more damaging to the enzyme's activity and/or stability than the deletion of an arginine residue in position 608. It follows that Type B patients who are homoallelic for ΔR608 would have a milder disease course than Type B patients who are heteroallelic for ΔR608 and a Type A mutation. Notably, proband 3 had comparatively milder disease manifestations than proband 2 at the same age. Thus, these genotype/phenotype correlations indicate that the more residual acid sphingomyelinase activity expressed by the mutant alleles, the milder the disease manifestations. Ideally, in vitro expression would permit comparisons of the activity and stability of the residual enzymes expressed by each mutant acid sphingomyelinase allele. However, it is likely that the residual activities expressed by the mutant alleles would be too low to permit biochemical characterization, particularly since eukaryotic expression systems (e.g., COS-1 cells, CHO cells) have endogenous acid sphingomyelinase activity and prokaryotic systems do not perform the posttranslational modifications (e.g., *N*-glycosylation) required for acid sphingomyelinase activity.

The occurrence of genetic heterogeneity resulting in dramatically different phenotypes is a hallmark of the lysosomal storage diseases. A notable example in which the molecular lesions have been correlated with distinct phenotypes is Tay-Sachs disease (26, 27). Mutations causing the classic, infantile form of Tay-Sachs disease have included deletions, insertions, and splice site mutations in the β-hexosaminidase A α-chain gene that resulted in no detectable transcripts, whereas an exonic point mutation expresses sufficient enzymatic activity to result in the adult or chronic type of GM_2 gangliosidosis. Analogously, identification of the R496L and ΔR608 mutations has provided the first insights into the molecular lesions in the acid sphingomyelinase gene underlying the remarkable phenotypic heterogeneity in Niemann-Pick disease. Clearly, individuals who are homoallelic for the R496L mutation will have a severe neuronopathic phenotype and Type A Niemann-Pick disease. The phenotype of patients heteroallelic for R496L will depend on the genetic lesion present on their other acid sphingomyelinase allele. In contrast, patients who are either homo- or heteroallelic for the ΔR608 (e.g., probands 2 and 3) will most likely have Type B Niemann-Pick disease. The identification of additional mutations causing Types A and B Niemann-Pick disease should permit reliable genotype/phenotype correlations and provide further insights into the functional organization of the acid sphingomyelinase polypeptide.

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