GM2-Gangliosidosis B1 Variant: Analysis of β-Hexosaminidase α Gene Mutations in 11 Patients from a Defined Region in Portugal

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Summary

The GM2-gangliosidosis B1 variant occurs at an exceptionally high frequency in the northern part of Portugal. In most patients, the disease manifests itself as a juvenile form, as opposed to the late-infantile form described for many patients from other parts of the world. We have analyzed the β-hexosaminidase α gene in 11 patients, as well as in some relatives, in order to characterize the underlying abnormalities. They were screened for the two previously identified mutations responsible for the B1 variant phenotype ($G^{533} \to A$, also designated as the "DN allele," and $C^{532} \to T$) by PCR amplification of an 800-bp DNA fragment and subsequent dot-blot hybridization with allele-specific oligonucleotides. The fragment amplified from one patient was also subcloned and sequenced. Ten patients, constituting a clinically and biochemically homogeneous group, were found to be homozygous for the DN allele. The other, whose clinical profile more resembled the late-infantile phenotype often described in the literature, was a compound heterozygote carrying the DN allele and another, as yet unidentified, abnormal allele. Our results, corroborated by previously published data, suggest that homozygotes and compound heterozygotes for the DN allele may be distinguishable at the phenotypic level, depending on the nature of the abnormality in the other allele. A common ancestral origin for the DN allele can also be postulated.

Introduction

GM2-gangliosidosis comprises a group of neurodegenerative disorders resulting from the genetically defective catabolism—and subsequent accumulation—of GM2-ganglioside (Sandhoff et al. 1989). Three major types are distinguished on the basis of the defective genes involved: the N-acetyl-β-hexosaminidase α-subunit, or β-subunit, and the natural activator protein, lead respectively, to the B variant (Tay-Sachs disease), the O variant (Sandhoff disease), and the AB variant.

The B1 phenotype is among the variant forms of the genetic β-hexosaminidase α defects. It is enzymologically unique in that β-hexosaminidase A is catalytically normal if tested with conventional artificial substrates but is inactive to hydrolyze natural GM2-ganglioside and sulfated artificial substrates (e.g., 4-MU-GlcNAc-6-sulfate) (Kytzia et al. 1983). Ohno and Suzuki (1988) described the first causal mutation in the α-subunit gene associated with the B1 phenotype in a Puerto Rican patient (DM). This point mutation, designated as the "DN allele," was a G-to-A transition at nucleotide 533 (counted from A of the initiation codon, ATG), translating into an arginine-to-histidine substitution at residue 178. Computerized molecular modeling of this region revealed drastic alterations in the three-dimensional structure of the mutant protein (Suzuki and Vanier, in press), consistent with the hypothesis that this region defines a functionally important domain of the α-subunit (Ohno and

Suzuki 1988). In subsequent studies, the same DN allele was found in six B1 variant patients of diverse ethnic and geographical origins (Tanaka et al. 1988), and a different point mutation (C^{532} to T) affecting the same codon (Arg^{178} to Cys) was identified in a Czechoslovakian patient (Tanaka et al. 1990). Except for a Spanish patient who was a DN-allele homozygote, all others were compound heterozygotes (Tanaka et al. 1990).

The finding of the wide distribution of the DN allele was somewhat surprising and was difficult to attribute to a coincidence in independent sporadic mutational events, even though it involves a CpG mutation hot spot. On the other hand, although three of these seven patients could be considered to be of "Hispanic" background (Puerto Rican, Spanish, and English/French/Azorean), it seemed unlikely that the allele had derived from a single ancestor. The present report describes a further study of 11 additional B1 variant patients from eight apparently unrelated families originating from a defined region in the north of Portugal. We hypothesize a common origin for this mutation, in view of both the unusually large number of homozygotes in this area and the migratory routes which link the Portuguese to some ethnicities of the previously described

cases. This work was presented in part at a meeting and was published in an abstract form (Dos Santos et al. 1990).

Patients, Material, and Methods

Patients

Altogether, 11 patients from eight different families were studied, including the seven patients described both above and elsewhere (Maia et al. 1990). The clinical and enzymological data on these patients are summarized in table 1. Patient 11 presented a clinical and biochemical profile of the late-infantile form of the disease, while all other patients were of the juvenile phenotype. The molecular studies also included parents and unaffected siblings in some families. Enzymatic assays were done as described elsewhere (Maia et al. 1990).

Lymphoblast Cultures

Epstein-Barr Virus (EBV)–transformed lymphoblast cell cultures were used as source material for DNA from all individuals. The cell lines were established starting from 5 ml of whole blood, according to

Table I

Clinical and Biochemical Summary of Patients

| | | | | β-Hexaminidase Activity[b] (nmol/h/mg protein) | | |
| | | | | 4-MU-GlcNAc | | Sulfated |
Case[a] (sex)	Initial Symptom	Age at Onset	Present Age or Age at Death	Total	% A	(Hex A)
1 (F)	Gait disturbance	3 years	11 years (deceased)	987	69.6	.99
2 (F)	Gait disturbance	3-4 years	12 years	1,340	76.3	.80
3 (F)	None (presymptomatic)	...	4 ½ years	1,220	70.1	2.90
4 (F)	Language delay	4-5 years	13 years (deceased)	914	60.6	.62
5 (M)	Gait disturbance	6 years	12 years	967	66.0	1.90
6 (M)	Speech loss	7 years	16 years	1,030	60.3	.59
7 (F)	Speech loss	4 years	9 years	1,010	66.0	.75
8 (M)	??	??	8 years	1,050	59.0	1.50
9 (F)	Gait disturbance		5 ½ years	763	67.6	2.70
10 (M)	Gait disturbance	3 years	10 years	785	54.0	1.96
11 (F)	Gait disturbance	14 mo	4 years	1,300	24.4	4.00

[a] Three pairs of patients—2 and 3, 6 and 7, and 9 and 10—are siblings. The parents of patients 9 and 10 are consanguineous. All families were from the following towns and villages, either in the district of Braga (cases 1–7) or in the district of Porto (cases 8–11), all of which are within 100 km from the city of Porto: Amarante (case 1), Braga (cases 2, 3, and 5), Vila Nova Famalicao (case 4), Celorico de Bastos (cases 6 and 7), Gondomar (case 8), Villa Nova Gaia (cases 9–11).

[b] Determined in leukocytes. The ranges of the control activities ($n = 54$) were 967–2,294 (total), 80.8–91.4 (% A), and 128–319 (sulfated substrate).

an modification of the method described by Neitzel (1986). EBV was harvested from the culture medium of cell line B95-8. Transformed lymphocytes were grown in RPMI-1640 (Roswell Park Memorial Institute series) medium + antibiotics + 10% inactivated FCS, which was supplemented with 2 μg cyclosporin A/ml during the first week following infection.

Screening with Allele-specific Oligonucleotides (ASO)

Genomic DNA was isolated from approximately 40 ml of saturated lymphoblast suspension cultures, according to the standard procedure (Maniatis et al. 1982). An approximately 800-bp fragment, including exon 5 of the β-hexosaminidase α gene, was amplified by the PCR with appropriate primers and was screened for both the DN allele and the Czechoslovakian allele by dot-blot analysis (Tanaka et al. 1990).

Sequencing

The amplified 800-bp fragment was digested with BamHI to generate two fragments, one of about 200 bp and one of about 600 bp. The mutation site is located on the larger fragment, approximately 100 bp downstream of the cleavage site. This fragment was subcloned into pUC19 and sequenced directly in the plasmid DNA by means of the dideoxy chain-termination method (Sanger et al. 1977), with ^{35}S-labeled dATP (Biggin et al. 1983), by using the 17-mer universal primer and T7 DNA polymerase.

Results

PCR amplification and dot-blot analysis with the three ASOs indicated that, in all patients except for patient 11, positive signals were obtained for the DN allele only (fig. 1). The phenotypically different patient 11 was clearly a compound heterozygote in that positive signals were present for both the normal allele and the DN allele (fig. 1). All other patients either were homozygous for the DN allele or were compound heterozygotes, possessing one DN allele and another abnormal allele with a mutation within the 21-bp region spanned by the ASOs, which was neither the DN nor the "Czechoslovakian" mutation. This ambiguity was resolved for most patients by examining both parents, who were found to be carriers of the DN allele, thereby allowing the confirmation of homozygosity in their offspring (fig. 1). In one patient (patient 5), whose parents could not be studied, sequencing was carried out on the amplified genomic fragment. Four consecu-
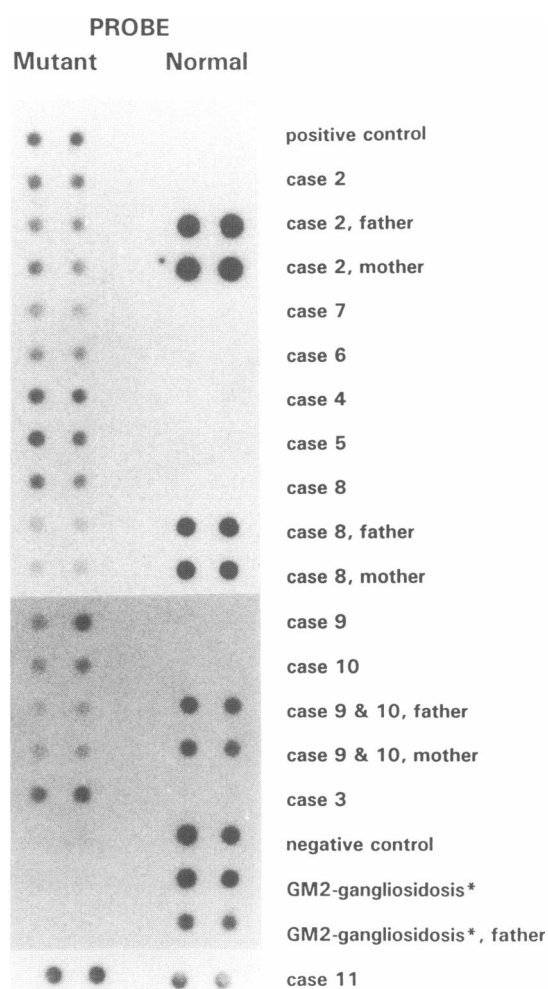

Figure I   Dot-blot analysis using allele-specific probes for DN mutation and for normal sequence. Samples are blotted in duplicate. Technical details are described in the text. Case numbers are as in table 1. The positive control is the Spanish patient who is homozygous for the DN allele (Tanaka et al. 1990), and the negative control is a normal individual. GM2-gangliosidosis* = patient with atypical but non–B1 variant GM2-gangliosidosis. Although not shown, the parents of patient 4 and those of patients 6 and 7 were also all carriers of the DN mutation. The parents of patient 5 were not tested, but four independent subclones of this patient all showed the DN mutation. Thus, patients 2–10 were all homozygous for the DN allele. In contrast, patient 11 is clearly a compound heterozygote with only one allele carrying the DN mutation. No individuals gave a positive signal for the Czechoslovakian mutation.

tive subclones all exhibited the DN mutation (results not shown). Although no biological material was available for one of the patients (patient 1), DNA samples were obtained from both of her parents and from her three sibs. Figure 2 shows that both parents carry
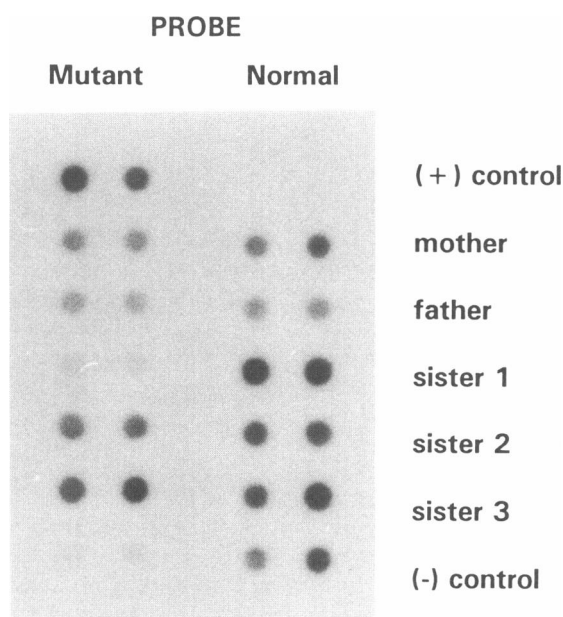
PROBE

Mutant   Normal

(+) control

mother

father

sister 1

sister 2

sister 3

(-) control

Figure 2   Dot-blot analysis of family members of patient 1 by using allele-specific probes for DN mutation and for normal sequence. Specimens were not available from patient 1. Thus, samples from the parents and from three unaffected siblings were screened according to the methods described in the text. The positive and negative controls are as in fig. 1. Both parents are carriers of the DN allele. Sister 1 is normal, while both sister 2 and sister 3 are carriers.

the DN mutation, and it was thus concluded that the deceased patient was also a DN-allele homozygote. These analyses also established the carrier status in two siblings, where previous biochemical studies had been inconclusive, because of overlapping of the value ranges obtained in control and carrier individuals fig. 2).

Discussion

Two causal mutations had previously been identified in patients presenting the B1 variant phenotype of G_{M2}-gangliosidosis (Tanaka et al. 1988, 1990). Both were single-base substitutions at the codon for Arg[178]. The DN allele, a G[533]-to-A mutation which was first identified in a Puerto Rican patient, was reported to be present in six of the seven patients studied. All were compound heterozygotes, except for a Spanish patient who was homozygous for the DN allele. Among the compound heterozygotes, the nature of the second mutant allele was identified only in the original Puerto Rican patient, who had the exon 11 four-base inser-

tion commonly associated with the infantile Jewish Tay-Sachs disease (Tanaka et al. 1990). In contrast, 10 of 11 patients in the present study were DN-allele homozygotes.

All 11 DN-allele homozygous patients (10 in the present study and the Spanish patient in Tanaka et al. 1990) were of the juvenile clinical phenotype with a late onset and slow progression. The original Puerto Rican patient, who had the DN mutation and an infantile mutation in the respective alleles, had a late-infantile form of the disease in that onset was at 11 mo, and death occurred at 4 years 8 mo. In the present study, the compound heterozygous patient (patient 11) showed a similar clinical profile, with onset at 14 mo and a rapidly progressive deterioration. Clinical phenotypes of the remaining compound heterozygous patients were variable. These observations suggest a genotype-phenotype correlation. In the homozygotes, the total residual activity of the mutant enzyme protein generated by the double dose of the DN allele presumably is sufficient for the juvenile phenotype. On the other hand, the clinical phenotype of DN-allele compound heterozygotes should depend on the nature of the second abnormal allele. When the second allele contributes no residual activity at all, such as in the original Puerto Rican patient, the disease will be of an earlier onset and more rapid progression but will not be as severe as in patients in whom neither allele generates residual activity, such as in classical infantile Tay-Sachs disease. If the second allele carries any mutation that can also contribute residual catalytic activity, the clinical phenotype can be expected to be correspondingly milder.

The exceptionally large number of B1-variant patients in a defined region of Portugal was unexpected for this presumably very rare disease. Consanguinity was found in only one of the eight families, which is indicative of an unusually high gene frequency in this area. A project is currently being undertaken to screen the local population by biochemical assays in urine samples (Ribeiro et al., submitted). Carriers detected in this way will subsequently be tested for presence of the DN allele by the oligonucleotide probes.

Considering that there is no positive record of relatedness among the families and that 10 patients are DN-allele homozygotes and that one is a DN-allele compound heterozygote, it appears that the mutation has been present in this population for some time. In keeping with the hypothesis of a common ancestral origin, the diverse migratory routes of the Portuguese could perhaps account for the mutation's wide geo-

graphic and ethnic distribution. The presence of the DN allele in at least some of the previously described cases—i.e., the Spanish patient (both parents originating from a Spanish village 15 km from the Portuguese border), the Puerto Rican patient (a large number of Portuguese emigrated to Puerto Rico around 1700), and the English/French/Azorean patient (whose maternal and paternal grandparents are from the Portuguese archipelago of Azores)—could be explained on this basis. Analysis of conserved sequences around the gene locus could contribute toward testing this hypothesis. Until such studies are undertaken, it is difficult to prove that in all instances the allele might have been inherited from a single ancestor. Meanwhile, this is considered a more plausible explanation for its wide distribution than ascribing the finding to numerous independent G^{533}-to-A mutations.

Acknowledgments

References

Biggin MD, Gibson TJ, Hong HF (1983) Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc Natl Acad Sci USA 80:3963–3965

dos Santos MR, Tanaka A, Ribeiro MG, Pinto RA, Maia M, sá Miranda MC, Suzuki K (1990) GM2 gangliosidosis B1 variant: clinical, biochemical and molecular studies. Paper presented at the International Workshop on Late Onset Neurometabolic Genetics Disorders, Siena, December 4–8

Kytzia H-J, Hinrichs U, Maire I, Suzuki K, Sandhoff K (1983) Variant of GM2-gangliosidosis with hexosaminidase A having a severely changed substrate specificity. EMBO J 2:1201–1205

Maia M, Alves D, Ribeiro G, Pinto R, Sá Miranda MC (1990) Juvenile GM2 gangliosidosis variant B1: clinical and biochemical study in seven patients. Neuropediatrics 21:18–23

Maniatis T, Fritch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Neitzel H (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. Hum Genet 73:320–326

Ohno K, Suzuki K (1988) Mutation in GM2-gangliosidosis B1 variant. J Neurochem 50:316–318

Ribeiro MG, Pinto RA, Dos Santos MR, Maia M, Sá Miranda MC. Biochemical characterization of β-hexosaminidase in different biological specimens from eleven patients with GM2 gangliosidosis B1 variant (submitted)

Sandhoff K, Conzelmann E, Neufeld EF, Kaback MM, Suzuki K (1989) The GM2-gangliosidosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 6th ed. McGraw-Hill, New York, 1807–1839

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Suzuki K, Vanier MT. Biochemical and molecular aspects of late-onset GM2-gangliosidosis: B1 variant as the prototype. Dev Neurosci (in press)

Tanaka A, Ohno K, Sandhoff K, Maire I, Kolodny EH, Brown A, Suzuki K (1990) GM2-gangliosidosis B1 variant: analysis of β-hexosaminidase α gene abnormalities in seven patients. Am J Hum Genet 46:329–339

Tanaka A, Ohno K, Suzuki K (1988) GM2-gangliosidosis B1 variant: a wide geographic and ethnic distribution of the specific β-hexosaminidase α chain mutation originally identified in a Puerto Rican patient. Biochem Biophys Res Commun 156:1015–1019