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A common β hexosaminidase gene mutation in adult Sandhoff disease patients

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Abstract β-Hexosaminidase gene mutations were analyzed in two adult-onset Sandhoff disease Italian patients by PCR analysis of a common known mutation (Δ5′) and by heteroduplex analysis of genomic and RT-PCR DNA fragments, covering the whole gene. The patients' genotypes were Δ5′/C1214T, and G890A/C1214T, respectively. As mutation C1214T (Pro405Leu) is also present in the other two late-onset cases so far described, we suggest that C1214T is a common mutation in this type of Sandhoff disease. Mutation G890A (Cys297Tyr) is a novel mutation which presumably causes altered processing of the pro β chain.

Introduction

Sandhoff disease (MIM 268800) is an autosomal recessive disorder caused by β-hexosaminidase (Hex) deficiency, which leads to storage of GM2-ganglioside and related asyaloglycolipids in neurons. Hex is a lysosomal hydrolase consisting of two major isozymes, Hex A and Hex B. Hex A is composed of an α and a β subunit, whereas Hex B is a homodimer of β subunits (Mahuran et al. 1985). Both subunits undergo post-translational modification converting the dimeric pro-Hex isozymes to their mature forms. The proα and the proβ polypeptides are similarly cleaved to produce the αp and βp chains. In addition, there is an internal cleavage unique to the proβ chain to produce the mature glycosylated polypeptides βb and βa, which are linked by a disulfate bond (Hasilik and Neufeld 1980; Mahuran et al. 1982; 1988). The two Hex subunits are encoded by separate genes on different chromosomes. The α subunit is encoded by the HEXA gene on chromosome 15 (Gilbert et al. 1975) and the β subunit is encoded by the HEXB gene on chromosome 5 (Lalley et al. 1974). While mutations in the HEXA gene cause deficiency in Hex A and determine Tay-Sachs disease, mutations at the HEXB locus produce deficiency in both isozyme activities and cause Sandhoff disease. Affected individuals display a wide spectrum of clinical symptoms and are usually classified as infantile, juvenile and adult forms, depending on the age of onset (Mahuran et al. 1985). Type I, the infantile form, is the most severe. It is characterized by the onset of symptoms during the first 6 months of life. Death generally occurs before 4 years of age. Type II, the juvenile form, is less severe. Symptoms appear later, at 3–10 years of age, and patients may survive into adulthood. A rare adult-onset form of the disease is also known (Oonk et al. 1979; Barbeau et al. 1984; Federico et al. 1986; Rubin et al. 1988). The disorder in adult-onset patients affects, at least at the beginning, mostly the motor neuron, cerebellar and autonomic functions. The human HEXB gene has been isolated and characterized; it is approximately 40 kb long and its coding sequence is split into 14 exons (Proia 1988). A few different mutations which cause Sandhoff disease have been identified. The most common one, which presently accounts for about 30% of the alleles (Bikker et al. 1990; Bolhuis and Bikker 1992), is a deletion at the 5′ end of the HEXB gene (Δ5′). This deletion removes approximately 16 kb of DNA including the HEXB promoter, exons 1–5 and part of intron 5 (Bikker et al. 1989; Neote et al. 1990). The presence of Δ5′ in the homozygous state determines an infantile onset of the disease. A C→T transition in the homozygous state was shown to produce the juvenile-onset form of Sandhoff disease in a Japanese patient. It is located at position 1214 of the cDNA (Pro405Leu), eight nucleotides downstream from the intron 10/exon 11 junction, and it inhibits proper mRNA splicing (Wakamatsu et al. 1992). The same mutation in heterozygosity with the Δ5′ mutation was also observed in an adult French-Canadian patient, indicating that the phenotype associated with the C1214T transition can vary from one individual to another (McInnes et al.

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1992). Here we report the characterization of two unrelated adult-onset Italian Sandhoff disease patients who were compound heterozygotes for the C1214T mutation. We also report the discovery of a novel HEXB gene mutation which modifies a Cys residue which is essential for the formation of an intramolecular loop involved in the maturation of the proβ chain.

Materials and methods

Patients

Patient 1 was previously described (Salviati et al. 1989). This 35-year-old man was evaluated at age 30 years because of slowly progressive lower limb weakness and diffuse fasciculations. He was an executive secretary and had been a rock climber until a year before, when lower limb weakness began. On neurological examination, moderate reduction of strength, slight bilateral wasting of hypothenar and thigh muscle, widespread spontaneous fasciculations and hyperactive tendon reflexes were observed. Intelligence was normal. CT scan of brain was normal. After 5 years of follow-up, the clinical picture is presently almost unmodified. Total Hex activity was reduced in serum and in leukocytes to 29.9 nmol/h per ml and 40.6 nmol/h per mg, respectively (normal values: > 350 nmol/h per ml and > 500 nmol/h per mg, respectively), the proportion of Hex A was pathologically elevated: 83.3% of total activity in serum and 77.8% in leukocytes (normal values: 53–75% in serum and 57–75% in leukocytes): Thin layer chromatography of urine revealed, after orcinol staining, small amounts of disaccharide. Rectal biopsy demonstrated, in autonomic neurons, the presence of storage material represented by membranous cytoplasmic bodies. Hex activity determinations revealed some carriers in the patient's family.

Patient 2 was also previously described (Federico et al. 1986). This patient was seen when he was 47 years old. At that age he presented a complex neurological syndrome, characterized by signs and symptoms of upper and lower motor neuron dysfunction, cerebellar ataxia, peripheral polyneuropathy, dysautonomia. Intelligence was normal. CT scan of brain was normal. Initial symptoms were represented by muscle fasciculations at about 20 years of age, followed, at age 32 years, by gait difficulties, cramps and paraesthesias. At the age of 34 years he noticed a decrease of libido, followed by autonomic nervous system dysfunction, with chronic diarrhea, hypohydrosis, impotentia and orthostatic hypotension. Total Hex activity was 79.9 nmol/h per ml in serum (Hex A = 99.5%) and 39.9 nmol/h per mg in leukocytes (Hex A = 95%). No oligosaccharides were present in urine, after thin layer chromatography and orcinol staining. Ultrastructural aspects of autonomic neurons in rectal biopsy were typical of gangliosidosis.

β-Hexosaminidase assay

Hex activity was determined using the artificial fluorogenic substrate 4-methyl-umbelliferyl-2-deoxy-2-acetamido-β-D-glucopyranoside (4-MU-GlcNAc) (Sigma). Heat-labile (Hex A) and heat-stable (Hex B) activities were determined in serum and in leukocytes by heat denaturation at 49 ± 0.1° C (Johnson et al. 1982).

Cell lines

Dermal fibroblast cultures were maintained at 37° C in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal calf serum.

DNA and RNA preparations

DNA was isolated from fibroblast cultures or peripheral blood by standard procedures. Total RNA was prepared from dermal fibroblast cultures by the guanidinium isothiocyanate method (Sambrook et al. 1989).

Detection of the Δ5′ mutation

The mutant Δ5′ allele in Sandhoff cell lines was detected by the polymerase chain reaction (PCR) procedure as described. Amplification of the deleted allele was achieved with primers flanking the deletion. The alleles not carrying the deletion were detected using primers located within the deletion in exons 4 and 5 (Neote et al. 1990).

Genomic and RT-PCR

First-strand cDNA was synthesized from 5 μg of total RNA with reverse transcriptase (RT; BRL) and 100 pmol of the pertinent reverse primer. An aliquot of the reaction mixture was used for PCR amplification through 25 cycles using *Taq* polymerase (Cetus Perkin Elmer). Four overlapping segments were amplified covering the coding sequence from part of exon 5 to exon 14 using primers described elsewhere (Dlott et al. 1990). Exons 1–5 were amplified from genomic DNA using primers described elsewhere (Zhang et al. 1994). Each cycle consisted of denaturation at 94° C for 1 min, annealing for 30 s between 50° C and 62° C depending on the primer set, and extension at 72° C for 30 s, followed by a final extension at 72° C for 5 min. In particular, for mutation C1214T detection, the amplified product included part of exon 8 (from nucleotide 894) to part of exon 11 (to nucleotide 1335); for mutation G890A detection, the amplified product was from part of exon 5 (from nucleotide 538) to part of exon 8 (to nucleotide 978); nucleotides are numbered according to Proia (1988).

Heteroduplex analysis

The PCR product from each patient was mixed in a 1 : 1 ratio with the PCR product from a normal individual. The mixture was heated to 95° C for 5 min, then cooled slowly to 37° C and incubated for 3 h at that temperature. Five microliters of this mixture were mixed with 1 μl of loading buffer and separated by electrophoresis in a 40-cm long and 0.8-mm thick Hydrolink-MDE gel.

Sequence determination

PCR products were adequately diluted and directly ligated to a PCR 1000 cloning vector (TA:A cloning system, Invitrogen), according to the manufacturer's instructions. Several independent clones were isolated and sequenced with the dideoxy chain-termination method using an Auto Read Sequencing kit (Pharmacia) on an A.L.F. DNA sequencer (Pharmacia).

Screening of mutations by restriction analysis

Genomic DNA from peripheral blood was prepared according to standard methods. A PCR product of 128 bp, containing the mutation C1214T, was obtained using primers F 5′ CATCTACTGTTCTAGGCCTA 3′ in intron 10, and R 5′ CAGATGCTGTGACTCTACTG 3′ in exon 11. The mutation abolishes a restriction site for the enzyme *Sca*F1. Restriction of the PCR product was for 3 h at 37° C. The mutation G890A of the HEXB coding sequence abolishes a restriction site for the enzyme *Nla*III. Genomic DNA was amplified using primers F 5′CCAGATCTTTATAATGAGT 3′ in intron 7, and R 5′ AGAGTCCAACTTGTTTTGTC 3′ in exon 8. Restriction was at 37° C for 3 h. DNA was analyzed in 15% polyacrylamide gels.

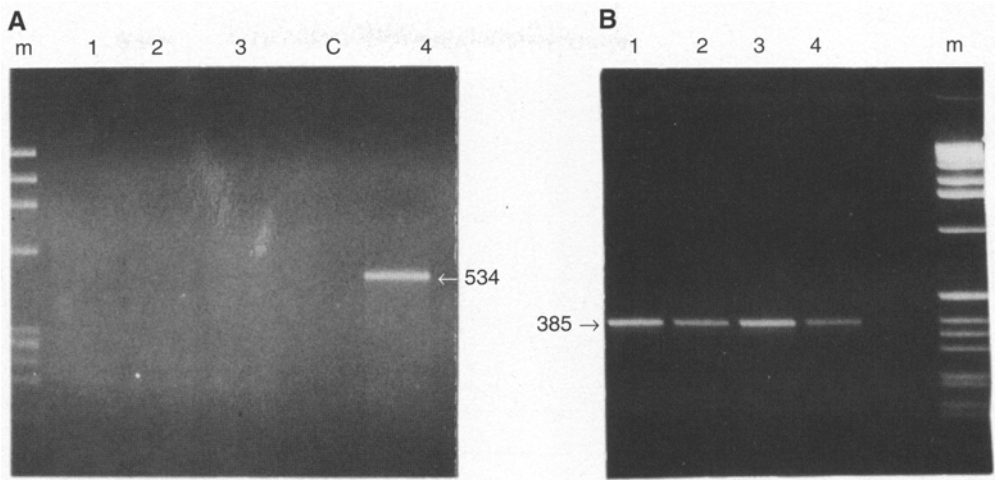

Fig. 1 A Detection of the Δ5′ of the HEXB gene in patient 1. Genomic DNA amplification using primers flanking the deletion. Lanes 1 and 2, two infantile-onset Sandhoff disease patients; 3 and 4, patients 2 and 1, respectively. C, control DNA; m, ϕX174 RF DNA digested with *Hae*III (BRL). **B** Genomic DNA amplification using primers within the deletion. Same samples as in **A** except m: 1 kb ladder, BRL. DNA fragment lengths in bp

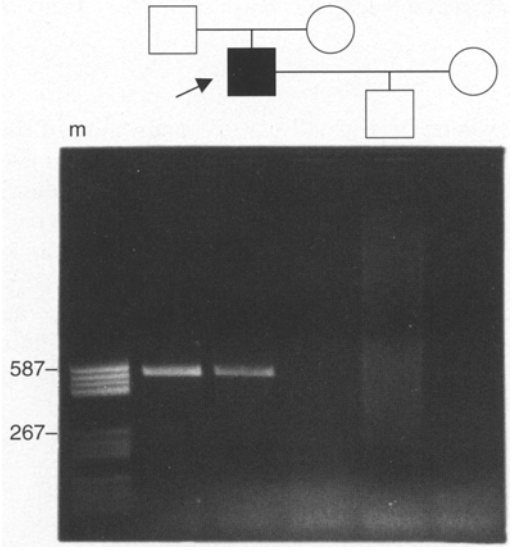

Fig. 2 Segregation analysis of Δ5′ in the family of patient 1 *(arrow)*. Genomic DNA amplification, using primers flanking the deletion. Lane m, molecular weight marker V (Boehringer Mannheim). The 534-bp amplified fragment indicates the presence of the deletion in the proband and his father

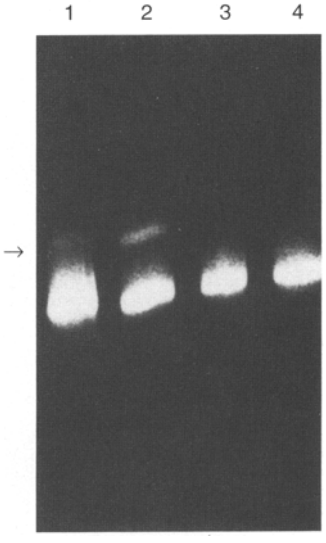

Fig. 3 Heteroduplex analysis of the RT-PCR product containing exons 8–11 of the HEXB gene in Sandhoff disease patients. Lanes 1 and 2, patients 1 and 2, respectively; 3, infantile-onset Sandhoff patient; 4, a normal control

Results

The Δ5′ mutation was amplified using primers flanking the deletion which frame a 534-bp sequence; the alleles not carrying the deletion were detected using primers contained within the deletion segment which give a product of 385 bp. In this way we have detected the presence of the Δ5′ deletion in patient 1 and we demonstrated that he is a genetic compound, since both the 534 (Fig. 1A, lane 4) and the 385-bp fragments were amplified (Fig. 1B, lane 4). Studying the patient's family we found that the Δ5′ allele was inherited from his father (Fig. 2). Sequence determination of the amplified product showed an exact correspondence of the deletion boundaries with the published sequence (Neote et al. 1990).

cDNAs from the patients and a normal control were amplified as described (Dlott et al. 1990) in four overlapping fragments, covering the coding sequence from exon 5 to exon 14. Exon 1–5 were directly amplified from genomic DNA according to Zhang et al. (1994). Amplified fragments from each patient and the control individual were mixed and subjected to heteroduplex analysis. In both patients, a 444-bp PCR product containing exons 8–11 produced heteroduplex bands (Fig. 3). This fragment was cloned and five or six independent clones were sequenced from each patient, respectively. Both patients were shown to carry one copy of the C1214T transition in exon 11, in all and in two clones, respectively (Fig. 4). In patient 1, the wild-type sequence was not present since the Δ5′ mutation does not lead to any transcript. No other discrepancy with the published sequence was observed in either patient. We observed that the mutation abolishes a *Scr*FI site in the HEXB gene: a DNA restriction analysis of some family members of patient 1 could be done to determine which of them carried the C1214T transition. Genomic DNA was amplified obtaining a 128-bp fragment. Restriction with *Scr*FI normally yields two fragments of 60 and 68 bp, respectively. Figure 5 indicates that the proband inherited the mutation from his mother and transmitted it to his son.

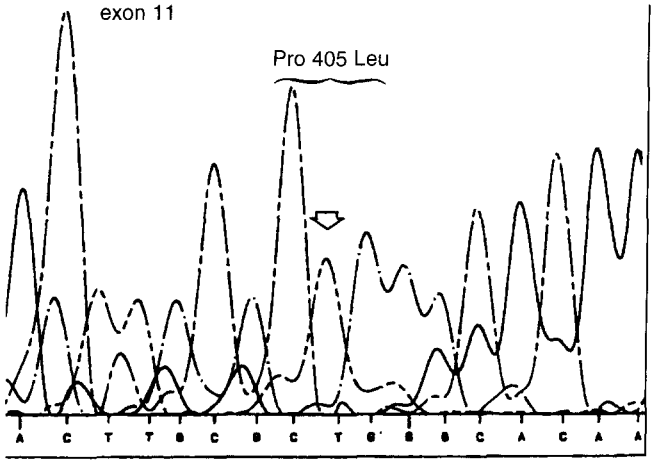

Fig. 4 Sequence determination of mutation C1214T of patient 1. The *arrow* indicates the nucleotide transition which determines the substitition of Pro405 with Leu in HexB

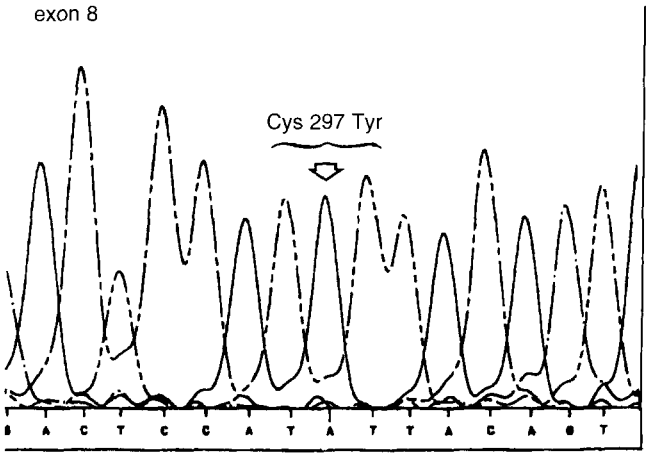

Fig. 6 Sequence analysis of the mutation G890A of patient 2. The *arrow* indicates the transition which substitutes Cys297 with Tyr in Hex B

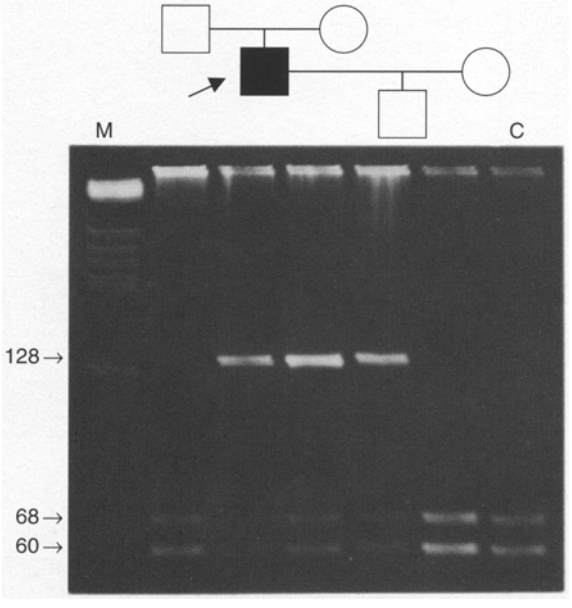

Fig. 5 Segregation analysis of the C1214T mutation in the family of patient 1 *(arrow)*. *Scr*F1 restriction of RT-PCR products. Lane M, molecular weight marker V (Boehringer Mannheim), C, normal control. The presence of the 128-bp fragment indicates heterozygosity for the mutation in the proband, his mother, and his child

Heteroduplex analysis of patient 2 showed an additional band in the fragment including exons 5–8 (data not shown). Sequence analysis of this fragment revealed a novel G890A transition (exon 8), which changes the triplet TGT corresponding to cysteine 297 into TAT, tyrosine (Fig. 6). To further exclude the possibility that this new alteration could represent a DNA polymorphism, genomic DNA was prepared from 50 healthy non-related individuals and a fragment of 111 bp of the HEXB gene, including the single-base mutation site, was amplified by PCR. The base substitution would result in the loss of the *Nla*III recognition site which normally yields two fragments of 83 and 28 bp, respectively. None of the 100 chromosomes from healthy individuals showed the presence of the transition G890A (data not shown).

Six unrelated infantile-onset Sandhoff patients (five Italian and one German) were also screened by restriction analysis for the C1214T or the G890A transition: none of them carried either mutation (data not shown).

The *trans* position of the two mutations present in patient 2 was demonstrated by amplification, cloning and sequencing of a 526-bp cDNA fragment including both mutation sites.

Discussion

In this study we report the complete genomic characterization of the HEXB gene mutations present in two unrelated Italian adult-onset Sandhoff patients, and indicate a possible common mutation in late-onset patients. Both patients were compound heterozygotes for the C1214T transition. This mutation inhibits normal HEXB mRNA splicing. Its causative role in Sandhoff disease has been demonstrated (Wakamatsu et al. 1992). In patient 1 (Δ5′/C1214T) the disease began at age 29 years with lower motor neuron dysfunction as the only clinical manifestation. Up to now, after 5 years of follow-up, neither other symptoms, nor signs of nervous system involvement have appeared. In patient 2, the other allele was a previously unreported G890A transition which causes a Cys297Tyr substitution. Given the correlation between the residual activity of the enzyme and the severity of the disease (Leinekugel et al. 1992), we propose that this mutation is severe, as it prevents the formation of a hydrophilic disulfide-loop structure between Cys297 and Cys348. Inside this loop, the proβ polypeptide is cleaved internally to produce the mature β_b and β_a chains (Sagherian et al. 1993). Loss of one disulfide bond in aspartyl-glucosaminidase, another lysosomal enzyme, results in early intracellular degradation of the enzyme (Ikonen et al. 1991).

The biochemical phenotype in patient 2 indicated an almost complete absence of Hex B activity, at least 95% of the residual Hex activity being represented by heat labile enzyme (Hex A and Hex S). In both patients, some catalytic activity in the Hex β subunit active site was probably present, since oligosaccharides were not detected in the urine of patient 2, and only a small amount of disaccharide was present in patient 1 (oligosaccharide hydrolysis is primarily accomplished by the active site of the β subunit of Hex A and Hex B).

This study confirms that the C1214T allele is associated with different late-onset phenotypes. This mutation was previously detected in two patients. The phenotype of a C1214T/C1214T 39-year-old Japanese patient (Wakamatsu et al. 1992) was characterized by a very slow clinical course, with onset in juvenile age of mental retardation and local panatrophy, probably the result of autonomic nervous system dysfunction. In a French-Canadian patient (McInnes et al. 1992), a C1214T compound heterozygote, as well as the two cases here described, onset was in adulthood, and intelligence was normal. In the Canadian patient at age 57 years symptoms and signs were restricted exclusively to the autonomic nervous system. In both our patients, the disease began clinically with signs of motor neuron dysfunction, loss of strength and fasciculations. At the same age, 34 years, however, patient 2 presented, in addition, severe signs of cerebellar dysfunction, dysautonomia and peripheral polyneuropathy, while patient 1 showed only an almost static lower motor neuron disorder.

It is difficult to explain these variations in phenotypic expression: genetic differences in the overall splicing efficiency between individuals have been proposed (McInnes et al. 1992). In addition, small variations in GM2-ganglioside concentration or distribution in different neuronal groups may be present, due to different rates of synthesis (Kracun et al. 1984). Other unknown hereditary or environmental factors may contribute.

In conclusion, the C1214T allele is present in a total of four out of four presently genotyped late-onset Sandhoff disease patients, suggesting that this may be the most frequent adult Sandhoff disease allele. A new mutation in the HEXB gene, GT890A, which probably destroys a disulfide bond, has been detected. We propose that this mutation has severe effects on enzyme activity and that the activity of the enzyme product of the C1214T allele in the genetic compound is enough to ensure the late onset of the disease.

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References

Barbeau A, Plasse L, Cloutier T, Paris S, Roy M (1984) Lysosomal enzymes in ataxia: discovery of two new cases of late onset hexosaminidase A and B deficiency (adult Sandhoff disease) in French Canadians. Can J Neurol Sci 11:601–606

Bikker H, van den Berg FM, Wolterman RA, Vijlder JJM, Bolhuis PA (1989) Demonstration of a Sandhoff disease-associated autosomal 50-kb deletion by field inversion gel electrophoresis. Hum Genet 81:287–288

Bikker H, van den Berg FM, Wolterman RA, Kleijer WJ, de Vijlder JJM, Bolhuis PA (1990) Distribution and characterization of a Sandhoff disease-associated 50-kb deletion of the gene encoding the human β-hexosaminidase β-chain. Hum Genet 85: 327–329

Bolhuis PA, Bikker H (1992) Deletion of the 5′-region in one or two alleles of the HEXB gene in 15 out of 30 patients with Sandhoff disease. Hum Genet 90:328–329

Dlott B, d'Azzo A, Quon DVK, Neufeld EF (1990) Two mutations produce intron insertion in mRNA and elongated beta-subunit of human beta-hexosaminidase. J Biol Chem 265:17921–17927

Federico A, Ciacci G, D'Amore I, Pallini R, Palmeris A, Rossi A, Rizzuto N, Guazzi GC (1986) GM2 gangliosidosis with hexosaminidase A and B defect: report of a family with motor neuron disease-like phenotype. J Inherited Metab Dis 9:307–310

Gilbert F, Kucherlapati R, Cregan RP, Murnane MJ, Darlington GJ, Ruddle FH (1975) Tay-Sachs's and Sandhoff's disease: the assignment of genes for hexosaminidase A and B to individual human chromosomes. Proc Natl Acad Sci USA 72:263–267

Hasilik A, Neufeld EF (1980) Biosynthesis of lysosomal enzymes in fibroblasts. Synthesis as precursors of higher molecular weight. J Biol Chem 255:4937–4945

Ikonen E, Enomaa N, Ulmanen I, Peltonen L (1991) In vitro mutagenesis helps to unravel the biological consequences of aspartylglucosaminuria mutation. Genomics, Sep; 11 (1:206–211)

Johnson WG, Wigger HJ, Karp HR, Glaubiger LM, Rowland LP (1982) Juvenile spinal muscular atrophy: a new hexosaminidase deficiency phenotype. Ann Neurol 11:11–16

Kracun I, Roesner H, Cosovic C, Stavljenic A (1984) Topographical atlas of the gangliosides of the adult human brain. J Neurochem 43:979–989

Lalley PA, Rattazzi MC, Shows TB (1974) Human beta-D-N-acetylhexosaminidase A and B: expression and linkage relationships in somatic hybrids. Proc Natl Acad Sci USA 71: 1569–1573

Leinekugel P, Michel S, Conzelmann E, Sandhoff K (1992) Quantitative correlation between the residual activity of β-hexosaminidase A and arylsulfatase A and the severity of the resulting lysosomal storage disease. Hum Genet 88:513-523

Mahuran DJ, Tsui F, Gravel RA, Lowden JA (1982) Evidence for two dissimilar polypeptide chains in the beta (2) subunit of hexosaminidase. Proc Natl Acad Sci USA 79:1602–1605

Mahuran D, Novak A, Lowden JA (1985) The lysosomal hexosaminidase isozymes. Isozymes. Curr Top Biol Med Res 12: 229–288

Mahuran DG, Neote K, Klavins MH, Leung A, Gravel RA (1988) Proteolytic processing of pro-alfa and pro-beta precursors from human hexosaminidase. J Biol Chem 263:4612–4618

McInnes B, Potier M, Wakamatsu N, Maloncon SB, Klavins MH, Tsuji S, Mahuran DJ (1992) An unusual splicing mutation in the HEXB gene associated with dramatically different phenotypes in patients from different racial background. J Clin Invest 90:306–314

Neote K, McInnes E, Mahuran DJ, Gravel RA (1990) Structure and distribution of an Alu-type deletion mutation in Sandhoff disease. J Clin Invest 86:1524–1531

Oonk JGW, van der Helm HJ, Martin JJ (1979) Spinocerebellar degeneration: hexosaminidase A and B deficiency in two adult sisters. Neurology 29:380–384

Proia RL (1988) Gene encoding the human β-hexosaminidase β chain: extensive homology of intron placement in the α- and β-chain genes. Proc Natl Acad Sci USA 85:1883–1887

Rubin M, Karpati G, Wolfe LS, Carpenter S, Klavins MH, Mahuran DJ (1988) Adult onset neuronpathy in the juvenile type of hexosaminidase A and B deficiency. J Neurol Sci 87:103-119

Sagherian C, Proszlay S, Vavougios G, Mahuran D (1993) Proteolytic processing of the proβ chain of β-hexosaminidase occurs at basic residues contained within an exposed disulfide loop structure. Biochem Cell Biol 71:340-347

Salviati A, Manganotti P, Tomelleri G, Fincati E, DeGrandi D, Passarin MG, Moretto G, Rizzuto N (1989) Typical motor neuron disease with hexosaminidase A and B deficiency. Ital J Neurol Sci 10:238

Sambrook J, Fritsch EF, Maniatis T (1989) Extraction of RNA with guanidinium thiocyanate followed by centrifugation in cesium chloride solutions. In: Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 7.19–7.22

Wakamatsu N, Kobayashi H, Myatake T, Tsuji S (1992) A novel exon mutation in human β-hexosaminidase β-subunit gene affecting the 3′ splice site selection. J Biol Chem 267:2406–2413

Zhang ZX, Wakamatsu N, Mules EH, Thomas GH, Gravel RA (1994) Impact of premature stop codons on mRNA levels in infantile Sandhoff disease. Hum Mol Gen 3:139–145