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Neurodegenerative lysosomal storage disease in European Burmese cats with hexosaminidase β-subunit deficiency

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ABSTRACT

GM2 gangliosidosis is a fatal, progressive neuronopathic lysosomal storage disease resulting from a deficiency of β-*N*-acetylhexosaminidase (EC 3.2.1.52) activity. GM2 gangliosidosis occurs with varying degrees of severity in humans and in a variety of animals, including cats. In the current research, European Burmese cats presented with clinical neurological signs and histopathological features typical of a lysosomal storage disease. Thin layer chromatography revealed substantial storage of GM2 ganglioside in brain tissue of affected cats, and assays with a synthetic fluorogenic substrate confirmed the absence of hexosaminidase activity. When the hexosaminidase β-subunit cDNA was sequenced from affected cats, a 91 base pair deletion constituting the entirety of exon 12 was documented. Subsequent sequencing of introns 11 and 12 revealed a 15 base pair deletion at the 3′ end of intron 11 that included the preferred splice acceptor site, generating two minor transcripts from cryptic splice acceptor sites in affected Burmese cats. In the cerebral cortex of affected cats, hexosaminidase β-subunit mRNA levels were approximately 1.5 times higher than normal ($P < 0.001$), while β-subunit protein levels were substantially reduced on Western blots.

β-*N*-acetylhexosaminidase (EC 3.2.1.52) is a lysosomal enzyme responsible for removal of the terminal *N*-acetyl-galactosamine residue from GM2 ganglioside. Functional hexosaminidase activity requires the coordinated action of three distinct proteins, a non-hydrolytic GM2 activator protein and the two hydrolytic subunits, α and β. Subunits may combine to form the two major hexosaminidase isozymes, each with different substrate specificities: HexB (ββ) or HexA (αβ), the isozyme responsible for degradation of GM2 ganglioside in humans. Thus, a defect in the α or β-subunit or in the GM2 activator protein causes GM2 gangliosidosis, classified as follows based on the remaining isozyme(s): variant AB (GM2 activator protein deficiency), variant B (α-subunit deficiency, Tay-Sachs Disease), or variant 0 (β-subunit deficiency, Sandhoff disease).

In addition to the human disease, GM2 gangliosidosis variant 0 has been reported in animals, including cats [1–3], pigs [4], dogs [5,6] and knockout mice [7,8]. Feline GM2 gangliosidosis is unique among large animal models of disease in the number and variety of mutations that have been reported. Pathogenic mutations include a 4 base pair deletion in the GM2 activator protein gene (*GM2A*) [9] and three distinct mutations in the β-subunit gene (*HEXB*), all of which produce a premature termination codon: a 25 base pair inversion at the 3′ terminus of the coding region [10], a single base deletion in exon 1 [11], or a nonsense mutation in exon 7 [12]. The current research reports a fourth distinct mutation of feline *HEXB*, a 15 base pair deletion that includes the splice acceptor site of intron 11, resulting in improper RNA splicing, removal of exon 12 from the mRNA and a premature termination codon. In addition, the deletion activates cryptic RNA splice sites in the vicinity of the mutation, generating novel transcripts not detected in normal cat tissue.

Materials and methods

Materials

Unless otherwise stated, standard laboratory reagents and fluorogenic enzyme substrates were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). 4-methylumbelliferyl-6-Sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside (MUGS) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Ganglioside standards for thin layer chromatography were purchased from Matreya (Pleasant Gap, Pennsylvania, USA). SuperScript II reverse transcriptase was purchased from Invitrogen (Carlsbad, California,

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USA), and SYBR green PCR core reagents were from Applied Biosystems (Foster City, California, USA). *Taq* DNA Polymerase was purchased from Fisher Scientific (Waltham, Massachusetts, USA). Secondary antibody and chemiluminescent substrate (Supersignal West Dura) were purchased from Thermo-Pierce (Rockford, Illinois, USA).

Enzyme assay

The enzyme activity of β-galactosidase, hexosaminidase, and α-mannosidase was measured with the corresponding 4-methylumbelliferone (4MU) substrate in cerebral cortex homogenates from two European Burmese cats showing severe neurological disease signs and normal cats not of European Burmese descent. β-galactosidase activity was measured by incubating 20 μl of homogenate with 0.5 mM 4MU-*N*-acetyl-β-D-glucosaminide, pH 3.8, for 60 min. For hexosaminidase A activity, 20 μl of sample was added to 1 mM MUGS, pH 4.2, for 60 min. Total hexosaminidase activity was tested by combining 5 μl of homogenate and 1 mM 4MU-*N*-acetyl-β-D-glucosaminide (MUG), pH 4.3, for 30 min. To assay α-mannosidase activity, 10 μl of sample was incubated with 2 mM 4MU-mannopyranoside, pH 4.2, for 60 min. After the allotted time, the enzyme activity was terminated with 3 ml of cold glycine carbonate buffer. Fluorescence of the 4MU compound was then measured on a Perkin-Elmer LS-5B luminescence spectrophotometer with excitation at 360 nm and emission at 450 nm. Protein concentrations were measured by the method of Lowry, and specific activity was expressed as nmol 4MU cleaved/mg protein/h.

Thin layer chromatography

High-performance thin layer chromatography was performed on brain tissue samples from affected European Burmese cats taken at necropsy and stored at −80 °C. The cortex samples (0.2–0.6 g) were homogenized in 2:1 chloroform:methanol and allowed to extract for 1 h at room temperature. The samples were centrifuged and the pellet extracted with 1:1 chloroform:methanol and again with 1:2 chloroform:methanol. Supernatants from each sample were then combined and dried under an air stream. The residues were treated with 3 ml of 0.1 N NaOH in methanol and incubated at 37 °C for 2 h and again dried under an air stream. The sample residues were then dissolved in 5 ml of ice cold water and neutralized, pH 4.5, with drop-wise addition of HCl. Lipids were further purified by reverse-phase column chromatography with a SEP-PAK C18 cartridge (Waters Associates Inc., Milford, Massachusetts, USA).

Extracted samples were applied to pre-coated silica gel 60 high-performance thin layer chromatography (HPTLC) plates (10 × 10 cm; Whatman, Florham Park, New Jersey, USA) which had been heat-activated for 30 min at 110 °C. Ganglioside standards and extracted samples were loaded onto the plate and developed with a chloroform–methanol-0.4% calcium chloride in water solution of 11:9:2. Plates were dried and sprayed with fresh resorcinol reagent, covered with clean glass plates, and heated for 20–30 min at 100 °C for color development.

Sequencing

Total RNA from frozen liver tissue of affected European Burmese cats was reverse transcribed using oligo-dT and SuperScript II at 50 °C for 1 h. The resultant cDNA was used as template for amplification of the 1700 base pair *HEXB* cDNA, sequenced in three overlapping segments as described previously for domestic shorthair cats [10]. Once the putative mutation was identified in the cDNA (deletion of exon 12), introns 11 and 12 were sequenced from genomic DNA of two known mutants (that exhibited clinical disease signs), one obligate heterozygote (confirmed by mating), and one normal cat from an unrelated feline breeding colony. Primers for intron amplification and sequencing were designed based on conserved exon/intron boundaries in humans and mice [13–15] and may be found in Table 1: Intron 11, 1338c and 1406n; Intron 12, 1387c and 1532n. To detect transcripts derived from cryptic splice acceptor sites in affected Burmese cats, nested PCR of the 5′ end of exon 12 was performed as follows. In round 1, primers 3U and 1378n [10] were used to amplify exons 9 (3′ end), 10, 11 and 12 (5′ end), followed by a second-round of nested PCR with primers 992c and 1378n to amplify minor transcripts containing exon 12 (Table 1). The PCR products were gel purified (Qiagen, Valencia, California, USA) and sequenced with amplification primers on an Applied Biosystems model 3100 Genetic Analyzer by the Auburn University Genomics and Sequencing Laboratory.

Diagnostic assay

After the putative mutation was identified in intron 11 of the feline *HEXB* gene, a diagnostic assay was developed using primers 1406n and Int11c (within intron 11, Table 1) to confirm the mutation and to genotype feline blood samples submitted by European Burmese breeders worldwide. Anti-coagulated blood samples (1 ml) in EDTA were submitted by overnight courier at ambient temperature for genomic DNA extraction and genotype analysis. Amplification was performed on a BioRad iCycler using *Taq* DNA polymerase with the following cycling parameters: initial denaturation at 95° C for 5 min followed by 38 cycles of 95° C for 45 s, 58° C for 45 s, and 72° C for 2 min, followed by a final elongation at 72° C for 6 min.

Table 1
Primers for investigation of European Burmese HEXB mutation.

Category	Fragment	Primer	Sequence (5′ → 3′)	Location[a]
Sequencing	Intron 11	1338c	AGT GGA CCC TCT TCA TTT TG	1333–1352
		1406n	AGA CAA GCT TCT CCA CCA ATG	1401–1381
	Intron 12	1387c	ATT GGT GGA GAA GCT TGT CTG T	1382–1403
		1532n	CTG TCA GTC TGT TGT AGG CAT T	1529–1508
	Exon 12 (Nested)	3U	TTT CCA GAT CAC TTT GTT CAC TTG	968–991
		1378n	GAC AAG CTT CTC CAC CAA TGA CA	1400–1378
		992c	GGA GGA GAT GAA GTG GAA T	992–1010
Diagnostic		Int11c	GCT GCC TCT TTT TGT GCC AA	Intron 11
		1406n	AGA CAA GCT TCT CCA CCA ATG	1401–1381
qRT-PCR	−Exon 12	3U	TTT CCA GAT CAC TTT GTT CAC TTG	968–991
		1040n	TCT GCT TCA TGA AAC CCT G	1058–1040
	+Exon 12	1280c	GCT TCC CTG TGA TCC TTT CTG CTC	1257–1280
		1378n	GAC AAG CTT CTC CAC CAA TGA CA	1400–1378

[a] Primers based on GenBank sequence S70340 (normal feline HEXB). Additional primers for sequencing feline HEXB can be found in the previous Ref. [10].