DISSERTATION

FELINE NIEMANN-PICK DISEASE TYPE C – THERAPEUTIC STUDIES AND GENETIC CHARACTERIZATION

Submitted by

Kyra Lynn Somers

Department of Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2000

Bell & Howell Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

COLORADO STATE UNIVERSITY

October 27, 2000

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KYRA LYNN SOMERS ENTITLED FELINE NIEMANN-PICK DISEASE TYPE C – THERAPEUTIC STUDIES AND GENETIC CHARACTERIZATION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Adviser

Department Chair

ABSTRACT OF DISSERTATION

FELINE NIEMANN-PICK DISEASE TYPE C – THERAPEUTIC STUDIES AND GENETIC CHARACTERIZATION

Niemann-Pick Disease Type C (NPC) is an autosomal recessive neurovisceral storage disorder in which defective intracellular transport of cholesterol results in lysosomal lipid accumulation. Clinical manifestations are heterogeneous, but usually include neonatal jaundice, onset and progression of neurological signs, hepatosplenomegaly, neurovisceral storage and premature death. The lysosomal storage in NPC involves a complex lipidosis characterized by accumulation of unesterified cholesterol, phospholipids and sphingomyelin. A feline model of NPC has been characterized and is clinically, biochemically, and morphologically equivalent to the major form of human NPC, NPC1. Chapters one and two of this dissertation describe therapeutic trials conducted in the feline model of NPC, while chapters three and four focus on the genetic characterization of the feline model of NPC.

Chapter one discusses the results of a diet study conducted in the feline model of NPC. The study goal of the cholesterol restricted diet was to lower the amount of low density lipoprotein (LDL) available to cells, hypothetically reducing subsequent lysosomal accumulation of unesterified cholesterol and other lipids. Results indicated liver lipid concentrations of unesterified cholesterol, cholesterol ester, and phospholipids in NPC affected treated kittens were similar to those seen in NPC affected untreated kittens. Ganglioside concentrations in the NPC affected treated kittens and NPC affected untreated kittens were also similar. Histologic findings in liver sections from NPC affected treated kittens showed a diffuse uniform microvacuolar pattern within hepatocytes and Kupffer cells, compared to a heterogeneous macro/micro vacuolar pattern and prominent nodular fibrosis in NPC affected untreated kittens. Similar differences in vacuolar patterns were seen in splenic macrophages. Neurological progression of disease was not altered and dietary cholesterol restriction did not significantly decrease storage in NPC affected treated kittens.

Chapter two describes the findings of a drug study, in which a potent ganglioside synthesis inhibitor, N-butyldeoxynojirimycin (NB-DNJ) was evaluated as a potential therapy for NPC. The predominant storage material outside of the central nervous system (CNS) includes unesterified cholesterol, phospholipids

and sphingomyelin. This is in contrast to the storage seen within the central nervous system, which is predominantly glycosphingolipids and in particular, gangliosides GM2 and GM3. A drug study was conducted to determine if NB-DNJ would prevent and/or slow disease manifestation in this feline model of NPC. Preliminary results from this study were encouraging with evidence of decreased ganglioside storage within the CNS and delayed clinical disease progression.

Chapter three summarizes complementation studies that were performed to determine if the gene responsible for the major form of human NPC and the feline model of NPC are orthologous. Cell fusions between human NPC and feline NPC fibroblasts were conducted to assess whether the multinucleated heterokaryons that were formed showed a reversal of the NPC phenotype. Cultured fibroblasts from NPC affected humans and NPC affected cats were hybridized and then analyzed for complementation by challenging the cells with LDL and subsequently staining with the fluorescent antibiotic filipin to visualize any abnormal accumulation of unesterified cholesterol. All of the multinucleated cells formed from the fusion of feline NPC fibroblasts and human NPC1 fibroblasts retained the NPC staining phenotype. This indicated an absence of

complementation and suggested that the underlying defect in the major form of human NPC, NPC1, and this feline model of NPC involve orthologous genes.

Chapter four describes the molecular genetic characterization of the feline model of NPC. Using human based PCR primers, initial fragments of the feline NPC1 cDNA were amplified and sequenced. Utilizing these sequences, feline specific PCR primers were generated and designed to amplify six bands which overlap and span the entire expected feline *NPC1* open reading frame. Ninety-eight percent of the feline NPC1 cDNA has been sequenced and a single base substitution (G2870C) has been identified in all NPC affected cats evaluated. Additionally, known carriers are heterozygous at the same allele. This mutation results in an amino acid change from cysteine to serine. The mutation site was utilized to design a PCR based assay for the identification feline NPC heterozygotes. Analysis to determine if cytogenetic abnormalities exist in NPC affected cats was performed. R-banded metaphase chromosome spreads of normal and known heterozygotes were generated. Karyotyping revealed that mutations resolvable at the cytogenetic level were not evident, supporting a small mutation. Utilizing FISH techniques, NPC1 PAC clones were used to determine the specific chromosomal location of NPC gene. Genetic characterization of feline NPC confirmed that the gene responsible for NPC in this feline model is

the orthologous gene that is mutated in the major form of human NPC, further substantiating the value of this colony as an animal model for NPC1.

Kyra Lynn Somers
Pathology Department
Colorado State University
Fort Collins, CO 80523
Fall 2000

ACKNOWLEDGEMENTS

The completion of this research and my graduate education was made possible by the contributions of many individuals whom I would like to acknowledge and thank. I am grateful for the guidance provided by my graduate committee members, Drs. Ramesh Akkina, Richard Bowen, Diane Brown, Duane Lassen, Pat Schultheiss and particularly, my adviser, Dr. Mary Anna Thrall.

I appreciate the support of the many members of the Thrall laboratory, both past and present, whose effort and dedication made the success of these studies possible. I am particularly grateful to Drs. Heather Connally, and Michael Royals. I would like to thank the staff at Laboratory Animal Resources for their assistance with veterinary care.

Thank you to Drs. Dwanye Hamar and Ronna Fulton for their expertise in the formulation of the low cholesterol diet. I would like to recognize other individuals who contributed to the diet study including Drs. Diane Brown, Pat Schultheiss, Robin Allison, and Cathy Just. Thank you to Drs Mary Smith and Karen R. Muñuna who generously provided their expertise and performed neurological

examinations for both the diet and drug studies. Thank you to Drs. Steve Walkley and Mark Zervas for their collaboration with the NB-DNJ drug study. Thanks to Oxford GycoSciences for generously donating the drug for the study and analyzing the serum samples. Dr. David Wenger provided cells for the complementation analysis and I would like to thank him. Many thanks to Drs. Gene Carstea and Jill Morris for providing the primers necessary to begin genetic characterization of the feline NPC model. Thank you to Mohammad Rafi for his help with developing the carrier assay. I would like to thank Dr. Elizabeth McNeil for karyotyping the NPC cats. Thank you to Dr. Stephen O'Brien and his laboratory for the development of the feline NPC PAC clones and for performing the FISH analysis. I offer thanks to Dr. Sherman Garver for his antibody work with the feline NPC protein. Special thanks to Dr. Thomas Mitchell who has performed countless necropsies, collected tissue samples and most importantly, has provided endless encouragement and support over the years.

I would like to thank the faculty and staff in the Pathology Department. Many of them have shared resources, offered advice and provided a positive atmosphere for personal and professional growth. I would also like to recognize and share my appreciation for the many cats that were a part of these studies.

I am eternally grateful for the love and support of my friends and my family. Without their encouragement, patience and understanding, this would have been a nearly impossible and a lonely endeavor.

This research was supported by NIH NIDDK grants RR06886 and DK09627.

DISSERTATION TABLE OF CONTENTS

DISSERTATION INTRODUCTION

Niemann-Pick Disease Type C (NPC) is an inborn error of metabolism characterized by lysosomal accumulation of lipids. Like many other lysosomal storage disorders, neurological abnormalities are present due to storage within the brain (1). Most lysosomal storage disorders result from a hydrolase deficiency, but that is not the case with NPC. Many several storage diseases have been identified in domestic animals (2). Development and characterization of animal models of lysosomal storage diseases has proven very useful in both the elucidation of pathophysiology and evaluation of therapeutic modalities.

NPC was first described in 1958 by Crocker and Farber. Increased tissue sphingomyelin was identified in a patient and the "new disease", NPC, was grouped with the other documented sphingomyelinase deficiencies, Niemann-Pick Diseases Type A and B (3). Niemann-Pick Disease Type C is now known to not be due to a sphingomyelinase deficiency, but the historical classification scheme persists (4, 5).

Niemann-Pick Disease Type C is an autosomal recessive inherited disease characterized by defective intracellular transport of exogenous, low density lipoprotein (LDL) derived cholesterol (6, 7). Uptake of LDL cholesterol is normal, but the NPC defect results in delayed cholesterol esterification and the subsequent accumulation of unesterified cholesterol that can be visualized by staining with the fluorescent antibiotic filipin (8, 9). The predominant storage material outside of the central nervous system (CNS) includes unesterified cholesterol, phospholipids and sphingomyelin. This is in contrast to the storage seen within the CNS, which is predominantly glycosphingolipids (GSL) and in particular, gangliosides GM2 and GM3. Ganglioside accumulation has been associated with ectopic dendritogenesis in several storage diseases(10). Cytological findings include vacuolated heptocytes and vacuolated macrophages in the both the spleen and bone marrow (11, 12).

Disease Manifestations of NPC

The clinical manifestations of human NPC include neonatal jaundice, hepatosplenomegaly, and a progressive neurodegenerative course marked by ataxia, dystonia, and dementia (13, 14). Niemann-Pick Disease Type C is panethnic and clinically heterogeneous, with varied ages of onset that are divided into acute (infantile), sub-acute (juvenile) and chronic (adult) categories (15). Prominent findings within the CNS include increased ganglioside accumulation, which is associated with the formation

of ectopic dendrites, (16) and axonal spheroid formation with subsequent neuoaxonal dytstrophy and cell death. GABAergic cells, including Purkinje cells in the cerebellum, are particularly affected (17). Recently, filipin staining of NPC neurons has shown storage of unesterifed cholesterol within neurons, contrary to earlier reports that cholesterol did not accumulate within the CNS of NPC patients (18, 19). Other abnormalities within the nervous system include a demyelinating and sensory polyneuropathy (20) in addition to the formation of neurofibrillary tangles (NFT's) consisting of paired helical filaments composed of tau proteins; these are similar to NFT's in Alzheimer's disease (21, 22).

Animal Models of NPC

Several models for NPC exist, including two murine models, the BALB/c LSD mouse and C57BL/KsJ (spm) mouse, in addition to the feline model that is the subject of this dissertation (9, 23). Both murine models have been shown to be genetic orthologs of the major form of human NPC, NPC1 (24, 25).

The feline model of NPC has been characterized and is phenotypically, morphologically, and biochemically identical to human NPC1 (17, 26-29). NPC affected cats typically present with clinical neurological signs between 8 and 12 weeks of age with rapid progression to severe dysmetria and ataxia, and death by 7 to 10 months of age.

Biochemical abnormalities include increased serum cholesterol and bilirubin concentrations, and increased serum alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase activities. Storage within the viscera is evident and includes vacuolization of hepatocytes, Kupffer cells, and macrophages. Within the CNS, axonal spheroids and ectopic dendrites are evident and by electron microscopy, membranous cytoplasmic inclusions are seen within neurons (27, 28).

Complementation studies using cultured fibroblasts from NPC affected cats and NPC1 affected humans suggested that the gene responsible for the NPC phenotype in this colony of cats is orthologous to the gene responsible for the major form of human NPC, NPC1 (30). Studies performed as part of this dissertation have since confirmed that the feline NPC gene is also an ortholog of the human NPC1 gene (Somers et al, unpublished data). Additionally, findings in feline NPC heterozygotes revealed morphological and biochemical abnormalities within the CNS, manifestations that have not yet been reported in carriers of genetic neuronal storage diseases (31).

Therapy for NPC

Currently, there is no effective treatment for NPC despite numerous attempts including bone marrow transplantation, cholesterol lowering diets and drugs, and the administration of ganglioside synthesis inhibitors (18, 32-36). Bone marrow transplants

have been performed in NPC cats and humans. Results for both species were similar and although there was a slight delay in clinical disease progression, the overall pattern of the disease course and subsequent premature death were not altered (32) (Brown et al, unpublished data).

Clinical trials in human beings using cholesterol lowering drugs and cholesterol restricted diets have had mixed results. In one study, dietary cholesterol restriction and treatment with cholesterol lowering agents, such as DMSO and lovastatin, were effective in decreasing plasma and hepatic cholesterol concentration; however, neurological function was not evaluated in one study, while no clinical benefit was noted in the other (33, 34). On the other hand, a single case study suggested that diet modification and cholesterol lowering agents might delay neurological deterioration on a short-term basis (35).

Most recently, treatment efforts have been targeted towards decreasing the abnormal ganglioside accumulation within the CNS. The predominant storage material outside of the CNS includes unesterified cholesterol, phospholipids and sphingomyelin, in contrast to the storage seen within the CNS, which is predominantly glycosphingolipids (GSL) and in particular, gangliosides (GM2 and GM3) (37-39). Ganglioside accumulation has

been associated with ectopic dendritogenesis in NPC, as well as in several other storage diseases, and may be a contributing factor to the neurological deterioration (40).

Molecular Bases of NPC

In Tay-Sachs and Sandhoff diseases, the predominant storage in the CNS is GM2 ganglioside (41-43). In two separate studies, N-butyldeoxynojirimycin (NB-DNJ) was used to treat Tay-Sachs or Sandhoff mice. Both studies showed NB-DNJ was successful in preventing the accumulation of GM2 within the brain (36, 44). Additionally, the Sandhoff mice showed delayed symptom onset, reduced storage in peripheral issues and increased life expectancy. Clinical studies are currently underway to treat type 1 Gaucher disease, a related disorder that involves GSL accumulation in peripheral tissues, but not the CNS (45).

These findings suggested that NB-DNJ might have a beneficial effect on GSL accumulation in the treatment of NPC and associated neurological dysfunction. NB-DNJ is an imino sugar and potent inhibitor of glycosphingolipid synthesis. NB-DNJ inhibits the N-linked oligosaccharide processing enzymes α-glucosidases I and II, and the ceramide specific glucosyltransferase that catalyses the first step in glucosphingolipid biosynthesis. The drug is water-soluble, is not metabolized and is excreted intact by the kidney (46-48). Treatment of HL-60 cells with NB-DNJ results in morphological changes

of secretory granules and the Golgi apparatus, both of which are non-cytotoxic and fully reversible upon withdrawal of the NB-DNJ (49). The drug is well tolerated by adult mice. However, when young mice were treated with NB-DNJ, extensive depletion of GSL within the liver and lymphoid organs was noted without any overt lesions, and the treated mice did grewmore slowly (50). The administration of a less toxic inhibitors such as N-butyldeoxygalactonojirimycin may avoid some of the negative side affects associated with NB-DNJ (51).

Other studies using double knockout mice revealed that the absence of ganglioside accumulation had no impact on the clinical NPC disease course and that visceral storage remained unchanged (18). NPC model mice were bred with mice carrying a mutated GalNAcT gene, which is responsible for the synthesis of GM2. No reduction in visceral storage was noted in the double mutants. However, they had significant reduction in the characteristic NPC brain lesions, including neuronal storage and decreased staining of neurons with filipin, but no improvement in the clinical NPC phenotype. Demyelination and neurodegeneration as determined by apoptotic cells and loss of Purkinje cells persisted as a prominent feature in the double mutants (18). These findings raise questions about the true role of ganglioside accumulation and the formation of ectopic dendrites as they relate to NPC and the characteristic neurological deterioration.

Only recently has the molecular basis of NPC begun to come to light. In 1993, Carstea et al first documented the linkage of NPC to human chromosome 18 (52). Complementation studies indicated that there were at least two genes that could be implicated in causing NPC (6, 53). The most common complementation group (major form) is now designated as NPC1 and accounts for greater than 95% of all NPC cases. The major form of human NPC (NPC1) has been mapped to chromosome 18q11; the human NPC gene (NPC1) has been identified and sequenced (24, 52). Studies also indicated that the gene responsible for the NPC phenotype in two murine models was linked to mouse chromosome 18, and homology studies showed that mouse chromosome 18 was syntenic with areas of human chromosomes 18 and 5(25). Over thirty different mutations of the NPC1 gene have been identified in humans (24, 54, 55). It has also been shown that Niemann-Pick disease type D (NPD), which arose from a founder effect in Nova Scotia, is an allelic variant of NPC (56).

The NPC1 gene codes for a 1278 amino acid protein. The putative NPC1 protein is predicted to have thirteen transmembrane regions and three large hydrophilic loops (57). The NPC1 protein has been shown to reside in late endosomes and to transiently interact with lysosomes and the trans-Golgi network (58). The exact function of the NPC1 protein remains unknown. However, it has been shown to be a transport protein that is critical for the movement of endocytosed material (58, 59). Several possible

functions of the NPC1 protein have been proposed and include 1) direct transporting that would facilitate the movement of cholesterol out of the lysosomes, 2) docking/fusion role that would mediate the interaction of cholesterol-laden vesicles and endosomes, or 3) functioning as a molecular pump (57). The mechanism by which the mutation in the NPC1 gene and resultant C957S amino acid substitution contributes to the pathogenesis of NPC remain unknown. The 957 cysteine residue is conserved across multiple species and is likely critical to the structure and subsequent function of the NPC1 protein (60).

The pathogenesis of the brain dysfunction in NPC is still not understood. It is clear that outside the central nervous system (CNS), the NPC1 lesion results from a processing defect of LDL-derived cholesterol, but it is widely accepted that exogenous cholesterol is not presented to the CNS because of the blood-brain barrier. This means that the NPC1 protein must have other functions, at least within the CNS, which have yet to be identified. Overall, the NPC1 protein is not closely related to any other known proteins, but the membrane orientation of the NPC1 sterol sensing domain (SSD) is identical to those of HMG CoA reductase and SCAP, known mediators of cholesterol homeostasis (61).

Characterization of the feline NPC1 cDNA sequence confirmed that the gene responsible for the NPC phenotype in the feline model is indeed the ortholog of the

human NPC1 gene. Strong nucleotide and amino acid sequence homology exists between the cat and human, which is actually greater than that between the human and mouse (Somers et al, unpublished data). This is not surprising given that comparative genomic studies have shown that the cat is phylogenetically more closely related to humans than the mouse, and that the overall feline-human syntenic chromosomal conservation is greater than the mouse-human (62). Based on these findings, the NPC feline colony will serve an extremely valuable model for further evaluation of the NPC1 protein and development of future treatment modalities.

Although the exact function of the NPC1 protein remains unknown, the field is rapidly advancing and it is probable that the elucidation of the function of the NPC1 protein will be determined in the very near future. These discoveries will play a pivotal role in understanding disease pathogenesis in NPC, in both the peripheral tissues and the CNS, and are vital to the development of rational treatment modalities.

The specific aims of the research presented in this dissertation were:

1) To evaluated dietary cholesterol restriction and ganglioside synthesis inhibitors as potential treatment modalities in the feline model of Niemann-Pick Disease Type C.

2) To characterize the gene responsible for the NPC phenotype in the feline model and to identify the mutation(s) in this cat colony.

REFERENCES

1. Walkley, S. U. (1998) Cellular pathology of lysosomal storage disorders. *Brain Pathol* 8, 175-193

2. Jolly, R. D., and Walkley, S. U. (1997) Lysosomal storage diseases of animals: an essay in comparative pathology. *Vet Pathol* 34, 527-548

3. Callahan, J. W., and Khalil, M. (1976) Sphingomyelinases and the genetic defects in Niemann-Pick disease. *Adv Exp Med Biol* 68, 397-378

4. de Winter, J. M., Janse, H. C., van Diggelen, O. P., Los, F. J., Beemer, F. A., and Kleijer, W. J. (1992) Prenatal diagnosis of Niemann-Pick disease type C. *Clin Chim Acta* 208, 173-181

5. Vanier, M. T., Pentchev, P., Rodriguez-Lafrasse, C., and Rousson, R. (1991) Niemann-Pick disease type C: an update. *J Inherit Metab Dis* 14, 580-595

6. Dahl, N. K., Daunais, M. A., and Liscum, L. (1994) A second complementation class of cholesterol transport mutants with a variant Niemann-Pick type C phenotype. *J Lipid Res* 35, 1839-1849

7. Liscum, L., Ruggiero, R. M., and Faust, J. R. (1989) The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick type C fibroblasts. *J Cell Biol* 108, 1625-1636

8. Pentchev, P. G., Comly, M. E., Kruth, H. S., Vanier, M. T., Wenger, D. A., Patel, S., and Brady, R. O. (1985) A defect in cholesterol esterification in Niemann-Pick disease (type C) patients. *Proc Natl Acad Sci U S A* 82, 8247-8251

9. Pentchev, P. G., Boothe, A. D., Kruth, H. S., Weintroub, H., Stivers, J., and Brady, R. O. (1984) A genetic storage disorder in BALB/C mice with a metabolic block in esterification of exogenous cholesterol. *J Biol Chem* 259, 5784-5791

10. Walkley, S. U., Siegel, D. A., Dobrenis, K., and Zervas, M. (1998) GM2 ganglioside as a regulator of pyramidal neuron dendritogenesis. *Ann N Y Acad Sci* 845, 188-199

11. Elleder, M., Smid, F., Hyniova, H., Cihula, J., Zeman, J., and Macek, M. (1984) Liver findings in Niemann-Pick disease type C. *Histochem J* 16, 1147-1170

12. Elleder, M., Hrodek, J., and Cihula, J. (1983) Niemann-Pick disease: lipid storage in bone marrow macrophages. *Histochem J* 15, 1065-1077

13. Brady, R. O., Filling-Katz, M. R., Barton, N. W., and Pentchev, P. G. (1989) Niemann-Pick disease types C and D. *Neurol Clin* 7, 75-88

14. Pentchev, P. G., Brady, R. O., Blanchette-Mackie, E. J., Vanier, M. T., Carstea, E. D., Parker, C. C., Goldin, E., and Roff, C. F. (1994) The Niemann-Pick C

lesion and its relationship to the intracellular distribution and utilization of LDL cholesterol. *Biochim Biophys Acta* 1225, 235-243

15. Vanier, M. T., Wenger, D. A., Comly, M. E., Rousson, R., Brady, R. O., and Pentchev, P. G. (1988) Niemann-Pick disease group C: clinical variability and diagnosis based on defective cholesterol esterification. A collaborative study on 70 patients. *Clin Genet* 33, 331-348

16. Walkley, S. U. (1995) Pyramidal neurons with ectopic dendrites in storage diseases exhibit increased GM2 ganglioside immunoreactivity. *Neuroscience* 68, 1027-1035

17. March, P. A., Thrall, M. A., Brown, D. E., Mitchell, T. W., Lowenthal, A. C., and Walkley, S. U. (1997) GABAergic neuroaxonal dystrophy and other cytopathological alterations in feline Niemann-Pick disease type C. *Acta Neuropathol (Berl)* 94, 164-172

18. Liu, Y., Wu, Y. P., Wada, R., Neufeld, E. B., Mullin, K. A., Howard, A. C., Pentchev, P. G., Vanier, M. T., Suzuki, K., and Proia, R. L. (2000) Alleviation of neuronal ganglioside storage does not improve the clinical course of the Niemann-Pick C disease mouse. *Hum Mol Genet* 9, 1087-1092

19. Vanier, M. T. (1999) Lipid changes in Niemann-Pick disease type C brain: personal experience and review of the literature. *Neurochem Res* 24, 481-489

20. Hahn, A. F., Gilbert, J. J., Kwarciak, C., Gillett, J., Bolton, C. F., Rupar, C. A., and Callahan, J. W. (1994) Nerve biopsy findings in Niemann-Pick type II (NPC) [see comments]. *Acta Neuropathol* 87, 149-154

21. Auer, I. A., Schmidt, M. L., Lee, V. M., Curry, B., Suzuki, K., Shin, R. W., Pentchev, P. G., Carstea, E. D., and Trojanowski, J. Q. (1995) Paired helical filament tau (PHFtau) in Niemann-Pick type C disease is similar to PHFtau in Alzheimer's disease. *Acta Neuropathol* 90, 547-551

22. Love, S., Bridges, L. R., and Case, C. P. (1995) Neurofibrillary tangles in Niemann-Pick disease type C. *Brain* 118, 119-129

23. Akaboshi, S., Yano, T., Miyawaki, S., Ohno, K., and Takeshita, K. (1997) A C57BL/KsJ mouse model of Niemann-Pick disease (spm) belongs to the same complementation group as the major childhood type of Niemann-Pick disease type C. *Hum Genet* 99, 350-353

24. Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B., Nagle, J., Polymeropoulos, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., Comly, M., Cooney, A., Brown, A., Kaneski, C. R., Blanchette-Mackie, E. J., Dwyer, N. K., Neufeld, E. B., Chang, T. Y., Liscum, L., Tagle, D. A., and et al. (1997) Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis [see comments]. *Science* 277, 228-231

25. Loftus, S. K., Morris, J. A., Carstea, E. D., Gu, J. Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M. A., Tagle, D. A., Pentchev, P. G., and Pavan, W. J. (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene [see comments]. *Science* 277, 232-235

26. Lowenthal, A. C., Cummings, J. F., Wenger, D. A., Thrall, M. A., Wood, P. A., and de Lahunta, A. (1990) Feline sphingolipidosis resembling Niemann-Pick disease type C. *Acta Neuropathol* 81, 189-197

27. Brown, D., Thrall, MA, Walkley, SU, Wenger, DA, Mitchell, TW, Allison, RW, Smith, MO, Royals, KL, March, PA. (1994) Animal Model of Human Disease: Feline Niemann-Pick Disease Type C. *Comparative Pathology Bulletin* XXVI

28. Brown, D. E., Thrall, M. A., Walkley, S. U., Wenger, D. A., Mitchell, T. W., Smith, M. O., Royals, K. L., March, P. A., and Allison, R. W. (1994) Feline Niemann-Pick disease type C. *Am J Pathol* 144, 1412-1415

29. Munana, K. R., Luttgen, P. J., Thrall, M. A., Mitchell, T. W., and Wenger, D. A. (1994) Neurological manifestations of Niemann-Pick disease type C in cats. *J Vet Intern Med* 8, 117-121

30. Somers, K. L., Wenger, D. A., Royals, M. A., Carstea, E. D., Connally, H. E., Kelly, T., Kimball, R., and Thrall, M. A. (1999) Complementation studies in human and feline Niemann-Pick type C disease. *Mol Genet Metab* 66, 117-121

31. Brown, D. E., Thrall, M. A., Walkley, S. U., Wurzelmann, S., Wenger, D. A., Allison, R. W., and Just, C. A. (1996) Metabolic abnormalities in feline Niemann-Pick type C heterozygotes. *J Inherit Metab Dis* 19, 319-330

32. Hsu, Y. S., Hwu, W. L., Huang, S. F., Lu, M. Y., Chen, R. L., Lin, D. T., Peng, S. S., and Lin, K. H. (1999) Niemann-Pick disease type C (a cellular cholesterol lipidosis) treated by bone marrow transplantation. *Bone Marrow Transplant* 24, 103-107

33. Patterson, M. C., Di Bisceglie, A. M., Higgins, J. J., Abel, R. B., Schiffmann, R., Parker, C. C., Argoff, C. E., Grewal, R. P., Yu, K., Pentchev, P. G., and et al. (1993) The effect of cholesterol-lowering agents on hepatic and plasma cholesterol in Niemann-Pick disease type C. *Neurology* 43, 61-64

34. Schiffmann, R. (1996) Niemann-Pick disease type C. From bench to bedside. *Jama* 276, 561-564

35. Sylvain, M., Arnold, D. L., Scriver, C. R., Schreiber, R., and Shevell, M. I. (1994) Magnetic resonance spectroscopy in Niemann-Pick disease type C: correlation with diagnosis and clinical response to cholestyramine and lovastatin. *Pediatr Neurol* 10, 228-232

36. Platt, F. M., Neises, G. R., Reinkensmeier, G., Townsend, M. J., Perry, V. H., Proia, R. L., Winchester, B., Dwek, R. A., and Butters, T. D. (1997) Prevention of

lysosomal storage in Tay-Sachs mice treated with N- butyldeoxynojirimycin. *Science* 276, 428-431

37. Brunngraber, E. G., Berra, B., and Zambotti, V. (1973) Altered levels of tissue glycoproteins, gangliosides, glycosaminoglycans and lipids in Niemann-Pick's disease. *Clin Chim Acta* 48, 173-181

38. Cruz, J. C., and Chang, T. Y. (2000) Fate of endogenously synthesized cholesterol in Niemann-Pick type C1 cells. *J Biol Chem*,

39. Elleder, M., Jirasek, A., Smid, F., Ledvinova, J., Besley, G. T., and Stopekova, M. (1984) Niemann-Pick disease type C with enhanced glycolipid storage. Report on further case of so-called lactosylceramidosis. *Virchows Arch A Pathol Anat Histopathol* 402, 307-317

40. Walkley, S. U., Zervas, M., and Wiseman, S. (2000) Gangliosides as Modulators of Dendritogenesis in Normal and Storage Disease-affected Pyramidal Neurons. *Cereb Cortex* 10, 1028-1037

41. Brownstein, S., Carpenter, S., Polomeno, R. C., and Little, J. M. (1980) Sandhoff's disease (GM2 gangliosidosis type 2). Histopathology and ultrastructure of the eye. *Arch Ophthalmol* 98, 1089-1097

42. Hoffman, L. M., Brooks, S. E., Amsterdam, D., Oropello, J., and Schneck, L. (1980) Tay-Sachs disease brain cells in culture: mobilization of stored GM2 after

concanavalin A-mediated uptake of hexosaminidase A. *J Neurosci Res* 5, 413-417

43. O'Brien, J. S. (1981) Ganglioside storage diseases: an updated review. *Ital J Neurol Sci* 2, 219-226

44. Jeyakumar, M., Butters, T. D., Cortina-Borja, M., Hunnam, V., Proia, R. L., Perry, V. H., Dwek, R. A., and Platt, F. M. (1999) Delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with N-butyldeoxynojirimycin. *Proc Natl Acad Sci U S A* 96, 6388-6393

45. Mistry, P. K. (2000) Treatment of Gaucher's disease with OGT 918 [letter]. *Lancet* 356, 676-677

46. Platt, F. M., and Butters, T. D. (1998) New therapeutic prospects for the glycosphingolipid lysosomal storage diseases. *Biochem Pharmacol* 56, 421-430

47. Platt, F. M., Karlsson, G. B., and Jacob, G. S. (1992) Modulation of cell-surface transferrin receptor by the imino sugar N- butyldeoxynojirimycin. *Eur J Biochem* 208, 187-193

48. Platt, F. M., Neises, G. R., Dwek, R. A., and Butters, T. D. (1994) N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. *J Biol Chem* 269, 8362-8365

49. Neises, G. R., Woodman, P. G., Butters, T. D., Ornberg, R. L., and Platt, F. M. (1997) Ultrastructural changes in the Golgi apparatus and secretory granules of

HL-60 cells treated with the imino sugar N-butyldeoxynojirimycin. *Biol Cell* 89, 123-131

50. Platt, F. M., Reinkensmeier, G., Dwek, R. A., and Butters, T. D. (1997) Extensive glycosphingolipid depletion in the liver and lymphoid organs of mice treated with N-butyldeoxynojirimycin. *J Biol Chem* 272, 19365-19372

51. Platt, F. M., Neises, G. R., Karlsson, G. B., Dwek, R. A., and Butters, T. D. (1994) N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing. *J Biol Chem* 269, 27108-27114

52. Carstea, E. D., Polymeropoulos, M. H., Parker, C. C., Detera-Wadleigh, S. D., O'Neill, R. R., Patterson, M. C., Goldin, E., Xiao, H., Straub, R. E., Vanier, M. T., and et al. (1993) Linkage of Niemann-Pick disease type C to human chromosome 18. *Proc Natl Acad Sci U S A* 90, 2002-2004

53. Steinberg, S. J., Ward, C. P., and Fensom, A. H. (1994) Complementation studies in Niemann-Pick disease type C indicate the existence of a second group. *J Med Genet* 31, 317-320

54. Greer, W. L., Riddell, D. C., Gillan, T. L., Girouard, G. S., Sparrow, S. M., Byers, D. M., Dobson, M. J., and Neumann, P. E. (1998) The Nova Scotia (type D) form of Niemann-Pick disease is caused by a G3097-->T transversion in NPC1. *Am J Hum Genet* 63, 52-54

55. Yamamoto, T., Nanba, E., Ninomiya, H., Higaki, K., Taniguchi, M., Zhang, H., Akaboshi, S., Watanabe, Y., Takeshima, T., Inui, K., Okada, S., Tanaka, A., Sakuragawa, N., Millat, G., Vanier, M. T., Morris, J. A., Pentchev, P. G., and Ohno, K. (1999) NPC1 gene mutations in Japanese patients with Niemann-Pick disease type C. *Hum Genet* 105, 10-16

56. Greer, W. L., Riddell, D. C., Byers, D. M., Welch, J. P., Girouard, G. S., Sparrow, S. M., Gillan, T. L., and Neumann, P. E. (1997) Linkage of Niemann-Pick disease type D to the same region of human chromosome 18 as Niemann-Pick disease type C. *Am J Hum Genet* 61, 139-142

57. Ioannou, Y. A. (2000) The Structure and Function of the Niemann-Pick C1 Protein. *Mol Genet Metab* 71, 175-181

58. Higgins, M. E., Davies, J. P., Chen, F. W., and Ioannou, Y. A. (1999) Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol Genet Metab* 68, 1-13

59. Neufeld, E. B., Wastney, M., Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Roff, C. F., Ohno, K., Morris, J. A., Carstea, E. D., Incardona, J. P., Strauss, J. F., 3rd, Vanier, M. T., Patterson, M. C., Brady, R. O., Pentchev, P. G., and Blanchette-Mackie, E. J. (1999) The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J Biol Chem* 274, 9627-9635

60. Watari, H., Blanchette-Mackie, E. J., Dwyer, N. K., Glick, J. M., Patel, S., Neufeld, E. B., Brady, R. O., Pentchev, P. G., and Strauss, J. F., 3rd (1999) Niemann-Pick C1 protein: obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. *Proc Natl Acad Sci U S A* 96, 805-810

61. Davies, J. P., and Ioannou, Y. A. (2000) Topological analysis of Niemann-Pick C1 protein reveals that the membrane orientation of the putative sterol-sensing domain is identical to those of 3-hydroxy-3-methylglutaryl-CoA reductase and sterol regulatory element binding protein cleavage-activating protein. *J Biol Chem* 275, 24367-24374

62. O'Brien, S. J., Wienberg, J., and Lyons, L. A. (1997) Comparative genomics: lessons from cats. *Trends Genet* 13, 393-399

CHAPTER ONE

DIETARY CHOLESTEROL RESTRICTION IN A FELINE MODEL OF NIEMANN-PICK DISEASE TYPE C

INTRODUCTION

The primary defect in Niemann-Pick Disease Type C (NPC) leads to abnormal processing of cholesterol, specifically exogenous cholesterol that enters cells via LDL receptor mediated pathways (1). This results in the accumulation of several lipids, including unesterified cholesterol. A diet study was conducted in this feline model of NPC to evaluate the effect of dietary cholesterol restriction on disease progression. The cholesterol restricted diet was targeted at reducing the amount of low density lipoprotein available to cells, hypothetically to prevent the subsequent accumulation of unesterified cholesterol in the lysosomes.

Clinical trials using low cholesterol diets in human beings have had mixed results. In one study, dietary cholesterol restriction and treatment with cholesterol lowering agents, such

as DMSO and lovastatin, were effective in decreasing plasma and hepatic cholesterol concentration (2,3). However, neurological function was not evaluated in one study, while no clinical benefit was noted in the other. On the other hand, a single case study suggested that diet modification and cholesterol lowering agents may delay neurological deterioration on a short-term basis (4). The aim of this study was to evaluate the impact of dietary cholesterol restriction on disease progression on disease progression in feline NPC. The hypothesis was that restriction of dietary cholesterol wouldl prevent cellular lipid accumulation and halt or delay clinical disease progression.

MATERIALS AND METHODS

Animals

Two NPC affected treated cats and two NPC affected non-treated cats were evaluated. Historical data from NPC affected non-treated cats were also used. Cats were fed controlled diets beginning at eight weeks of age and were maintained for 150 and 180 days respectively. The cats were individually housed and fed twice daily; the weight of food consumed was recorded. Cats were cared for according to the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, under the guidelines of the Institutional Animal Care and Use Committee (ACUC), and housed in a facility certified by the American Association for Accreditation of Laboratory Animal Care (AAALAC) at Colorado State University. At the end of study, animals were

euthanized with an overdose of intravenous barbituate (40mg/lb pentobarbital), complete necropsies were performed and tissues for analyses were collected.

Cholesterol Restricted Diet

A cholesterol restricted diet, formulated in conjunction with veterinary nutritionists and a commercial pet food company (Ralston Purina™), contained less than 2.6 mg of cholesterol per 100 grams of diet on a dry matter basis (Table 1). The diet was soybean based and included menhaden fish oil as the only source of cholesterol. (Cats are true carnivores and require an exogenous arachidonic acid source (menhaden fish oil), precluding a cholesterol free diet). The cholesterol restricted diet was mixed and stored as a powder at 4°C then mixed with water just prior to feeding. The control diet included two commercial pet foods (dry Meow Mix™ and canned Science Diet™ Feline Growth) containing 41.7 mg and 484.4 mg of cholesterol per 100 grams of diet on a dry matter basis respectively. Cholesterol content of all diets was determined by gas separation chromatography at Woodson-Tenent Laboratories (Des Moines, IA, USA).

Clinical Neurological Assessment

Neurological examinations of NPC affected treated and untreated cats were conducted every two weeks beginning at eight weeks of age. Standard evaluation of postural, reflex

and cranial nerve function was performed by a board certified veterinary neurologist who was unaware of the treatment status of individual cats.

Serum Biochemical Analyses

Cats were bled for serum biochemical analyses by jugular venipuncture following a 12 hour fast. Serum chemistry analyses were performed on an automated analyzer (Hitachi 704 or 911; Boehringer-Mannheim Corporation, Indianapolis, IN, USA). Analytes included serum bilirubin, cholesterol and albumin concentrations, and serum alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities. Serum for biochemical analyses was collected every four weeks beginning at two weeks of age.

Liver Lipid Analyses

Analyses of unesterified cholesterol, cholesterol ester and phospholipids in liver tissue were performed at the end of the study. Lipid extraction of fresh frozen liver was performed by the method of Folch (5). Briefly, a known weight of liver was extracted with chloroform-methanol (2:1 v/v) and filtered; 1/5 volume of water was added to the filtrate. Phases were separated by centrifugation and the lower phase was used for quantification of individual lipids. Total and individual lipids were quantified using standard methods (6) and expressed as nmol lipid/g wet weight tissue.

Brain Ganglioside Analyses

Cerebral cortex was collected at necropsy and frozen at –70°C. Gangliosides were extracted and purified by modified Suzuki method (7) and separated by high performance thin layer chromatography (HPTLC). Plates were scanned by densitometry (Quick-Scan Flur-Vis densitometer, Helena Laboratories, Beaumont, Texas, USA); G_{M1}, G_{M2}, and G_{M3} gangliosides were quantified.

Morphological Analyses

Light microscopic examination of liver, spleen and brain was performed at the end of the study. Tissues were collected at necropsy, fixed in 10% neutral buffered formalin, processed routinely, sectioned at 6µm, and stained with hematoxylin and eosin (H&E). Cerebellar tissue sections stained with H&E were quantitatively assessed by enumeration of Purkinje cells (CNIH Image software, version 1.61, W. Rasband, National Institutes of Health, Bethesda, MD) and counting of axonal spheroid numbers per 40X objective field averaged over eight fields.

Statistical Analysis

The number of NPC affected treated cats was inadequate for statistical comparison. Data for the two NPC affected treated cats are actual values while data for NPC affected untreated cats are means with standard deviation or ranges.

RESULTS

Clinical Neurological Assessment

Age of onset and rate of progression of neurological disease in the two NPC affected cats fed the cholesterol restricted diet were not different from NPC affected untreated cats. In general, NPC affected cats display intention head tremors between 8 and 12 weeks of age with a mean onset at 10.25 weeks of age. The NPC affected treated cats in the study showed neurological signs at 8 and 9 weeks of age and progressed neurologically at the same rate as NPC affected untreated cats. The intention tremors became more pronounced over time. Dysmetria during ambulation and hypermetric limb movements with truncal swaying were noted. By 19 to 23 weeks of age, NPC affected cats maintained a crouched position and would fall to either side when moving. Slow initiation and hypermetria of postural reactions were noted, and intention tremors had progressed to whole body involvement. Between the ages of 20 and 33 weeks, cats were unable to right themselves from lateral recumbency. Overtime, the tremors became less apparent, cats developed disuse muscle atrophy and menace response was lost. Both treated and untreated cats were euthanized between 7 and 9 months of age.

Serum Biochemical Analyses

Previously identified biochemical abnormalities in the feline model of NPC include increased serum ALP, ALT, and AST activities, increased serum bilirubin and cholesterol

concentrations and decreased serum albumin concentration (8,9). Serum abnormalities are not present in feline NPC heterozygotes (10).

Serum ALP activity and cholesterol concentration were lower in one NPC affected treated cat, as compared to NPC affected untreated cats from 12 weeks of age until the last sampling, and were comparable to values seen in the normal cats. Serum albumin concentrations were higher in both NPC affected treated cats compared to NPC affected untreated cats and similar to those seen in normal cats. Both NPC affected treated kittens had normal serum bilirubin concentrations. Serum biochemical data are summarized in Table 2.

Tissue Lipid Analyses

Liver lipid concentrations in NPC affected treated cats were similar to those in NPC affected untreated cats (Table 3). Ganglioside concentrations of G_{M1}, G_{M2}, and G_{M3} in the cerebral cortical grey matter of the NPC affected treated cats remained unchanged when compared to ganglioside concentrations in NPC affected untreated cats (Table 4).

Morphological Analyses

Hepatocyte cytoplasm was vacuolated in both the NPC affected treated and NPC affected untreated cats. In the NPC affected treated cats, cytoplasmic vacuolar changes

were diffuse and uniform throughout each hepatic lobule; all hepatocytes were swollen and rounded. The vacuoles were small, round, clear, and of almost uniform diameter. Occasional cells had larger vacuoles. By contrast, in the NPC affected untreated cats, cytoplasmic vacuoles were markedly variable in size and were usually larger than those seen in the NPC affected treated cats (Figure 1.1, 1.2). In the NPC affected untreated cats, narrow bands of fibrous tissue were present in the limiting plates of the lobules and extended through the lobules in a random fashion, disrupting the orderly arrangement of the hepatic cords. Fibrosis was not evident in NPC affected treated cats. Individual hepatocytes were occasionally surrounded by thin bands of collagen. Hepatocytes near the collagenous bands were smaller than more distant hepatocytes or those in the NPC affected treated cats (Figure 1.3, 1.4).

Splenic macrophages had similar differences in vacuolization (Figure 1.5, 1.6). Macrophages in the NPC affected treated cats contained many small uniform clear cytoplasmic vacuoles, while in the NPC affected untreated cats, vacuoles were more variable in size, with larger cytoplasmic vacuoles predominating.

Purkinje cell numbers decrease with age in all NPC affected cats (11). Cerebellar Purkinje cell concentration in both NPC affected treated and untreated cats was similar, but reduced compared to that of normal cats. The number of Purkinje cells seen in NPC

affected untreated cats was 1.1 (SD ± 0.54) per 100μm^2, compared to the two NPC affected treated cats, which were1.3 and 0.7 per 100μm^2 respectively. Normal control cats had a mean of 2.0 (SD ± 0.48) Purkinje cells per 100 μm^2. Large round hypereosinophilic axonal spheroids were prominent in the cerebellar granular and Purkinje cell layers in both NPC affected treated cats and NPC affected untreated cats (Figure 1.7, 1.8). A mean of 1.4 (SD ± 0.27) axonal spheroids per 40X objective field was seen in the NPC affected untreated cats, compared to 1.6 and 1.4 in the two NPC affected treated cats. No axonal spheroids were seen in normal control cats.

DISCUSSION

Dietary cholesterol restriction did not significantly impact disease progression in the two NPC affected treated cats in this study. Hepatic parameters, particularly serum albumin and cholesterol concentrations and serum ALT and ALP activity, appear to have improved in one kitten. The apparent improvement in these parameters, as well as the change in vacuolar patterns in the liver and spleen sections of the NPC affected treated cats, may be related to alterations in liver lipid metabolism following dietary cholesterol restriction. However, other dietary variables such as phytosterols and vitamin E content may have contributed to the results. Phytosterols are present in high concentrations in

soybeans and are known to reduce cholesterol absorption (12). Vitamin E has been shown to be beneficial in lowering serum lipid concentrations. Despite the potential presence and beneficial impact of other cholesterol attenuating substances in the diet, no significant change in disease progression was seen.

It appears that reduced dietary cholesterol has little impact on brain lesions and ganglioside storage was not decreased in NPC affected treated cats. Perhaps this is related to the fact that while visceral tissues store unesterified cholesterol and phospholipids in addition to glycolipids, NPC affected brains store predominantly gangliosides (G_{M2} and G_{M3}). Ganglioside synthesis inhibitors such as N-butyldeoxynojirimycin have shown promise as a potential therapy for NPC (13), Other studies involving double mutant mice (NPC model mice crossed with mice unable to synthesize G_{M2} and complex gangliosides) showed that the absence of ganglioside storage in the central nervous system (CNS) had no impact on the clinical NPC course of the disease (14). These finding suggested that ganglioside storage may be a secondary event and the primary mechanism for neurological dysfunction in NPC likely remains to be identified.

This study would have been improved by increasing the number of affected treated cats, including normal treated controls and starting the diet at an earlier age, but there was a

very limited supply of NPC affected kittens and the diet would have been difficult to administer prior to weaning of kittens (eight weeks of age). Earlier weaning is not well tolerated by NPC affected kittens, which tend to not do well in general. Although the number of animals evaluated may be too small to draw definitive conclusions, dietary cholesterol restriction did not impact disease progression in these two NPC affected treated cats. These findings are important and consistent with studies in people, suggesting the key to treating NPC lies elsewhere and that other treatment modalities need to be explored. This animal model may be valuable for future studies of intracellular cholesterol metabolisms and the development and evaluation of novel treatment modalities for NPC.

REFERENCES

1. Blanchette-Mackie EJ, Dwyer NK, Amende LM, et al (1988) Type-C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes. *Proc Natl Acad Sci USA*85:8022-8026

2. Patterson MC, Bisceglie D, Higgins JJ, et al (1993) The effect of cholesterol-lowering agents on hepatic and plasma cholesterol in Niemann-Pick disease type C. *Neurology* 43:61-64.

3. Schiffman R (1996) Niemann-Pick disease type C: From Bench to Bedside. *JAMA* 276:561-564.

4. Sylvain M, Arnold D, Scriver C, et al (19940 Magnetic resonance Spectroscopy in Niemann-Pick disease type C: Correlation with diagnosis and clinical response to cholestryamine and lovastatin.

5. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497-509.

6. Wenger DA, Sattler M, Kudoh C et al (1980)Niemann-Pick Disease: a genetic model in a Siamese cat. *Science* 208:1471-1473.

7. Ledeen RW, Yu RK (1982) Ganglioside Structure: isolation and analysis. *Methods Enzymol* 83:39-191.

8. Brown DE, Thrall MA, Walkley SU, et al (1994a) Feline Niemann-Pick Disease Type C. *Am J Pathol* 144:1412-1415.

9. Brown DE, Thrall MA, Walkley SU, et al (1994b) Animal Model of Human Disease: Feline Niemann-Pick Disease Type C. *Comp Pathol Bull* 27: 4-6.

10. Brown DE, Thrall MA, Walkley SU, et al (1996) Metabolic abnormalities in feline Niemann-Pick type C heterozygotes. *J Inher Metab Dis* 19:1-12.

11. March PA, Thrall MA, Brown DE, et al (1997) GABAergic neuroaxonal dystrophy and other cytopathological alterations in feline Niemann-Pick disease type C. *Acta Neuropath* 94:164-172.

12. Ling WH, Jones PJ (1995) Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sciences* 57:195-206.

13. Zervas M, Somers K, Thrall MA, et al (1999) Inhibition of Ganglioside Synthesis in Murine and Feline Models of Niemann-Pick Disease Type C ameliorates Neurological Disease. International Niemann-Pick C Disease Symposium. NIH, Bethesda, MD, October 1999, Abstract #79

14. Liu Y, Wu YP, Wada R, et al (2000) Alleviation of neuronal ganglioside storage does not improve the clinical course of the Niemann-Pick C disease mouse. *Hum Mol Genet* 9:1087-1092.

Table 1

Composition of Cholesterol Restricted Diet

Dietary Component	Proportion of Diet (%)
Soy Bean Meal	50.00
Wheat Germ Meal	16.00
Canola Oil	25.00
Menhaden Fish Oil	0.15
Molasses	4.20
Purina ™Mineral Mix	0.20
Purina™ Vitamin Mix	0.70
Taurine	0.10
Calcium Oxide	0.80
Potassium Phosphate	2.54
Zinc	0.03
Sodium Chloride	0.20
Methionine	0.08

Table 2

Serum Biochemical Analytes

Serum Analyte	AUTC (n=12)		S22	S96	Normal (n=32)	
	Mean	Range			Mean	Range
Alkaline Phosphatase (IU/L)	500	310-670	370	230	230	120-340
Alanine Aminotransferase (IU/L)	310	230-400	500	215	70	40-95
Cholesterol (mg/dl)	210	160-310	180	145	105	60-175
Bilirubin (mg/dl)	0.3	0.2-0.4	0.1	0.2	0.1	0-0.2
Albumin (gm/dl)	2.7	2.6-2.8	3.3	3.4	3.1	2.3-3.9

AUTC – NPC affected untreated control

S22, S96 – NPC affected treated (actual values)

Normal – untreated controls

Table 3

Liver Lipid Concentrations (nmol/g wet tissue weight)

Lipid	AUTC (n=6)		S22	S96	Normal (n=14)	
	Mean	Range			Mean	Range
Unesterified Cholesterol	45,800	31,000-72,550	66,600	47,525	3900	1200-6800
Cholesterol Ester	760	150-2350	3,680	690	2000	500-6300
Total Phospholipids	46,100	35,840-60,800	56,660	61,390	25,400	11,870-28,780
Sphingomyelin	5790	3200-11380	12,330	10,790	1150	100-2200
Phosphatidycholine	10,600	4300-15,700	11,230	9900	4670	2280-6240

AUTC – NPC affected untreated control

S22, S96 – NPC affected treated (actual values)

Normal – untreated controls

Table 4

Cerebral Cortex Ganglioside Concentrations (μg/mg of tissue)

Ganglioside	AUTC (n=6)		S96	Normal (n=11)	
	Mean	Range		Mean	Range
GM1	0.068	0.037-0.125	0.048	0.118	0.34-0.137
GM2	0.034	0.011-0.083	0.036	0.010	0.008-0.026
GM3	0.038	0.002-0.124	0.029	0.002	0.0-0.009

AUTC – NPC affected untreated control

S96 – NPC affected treated (actual values)

Normal – untreated controls

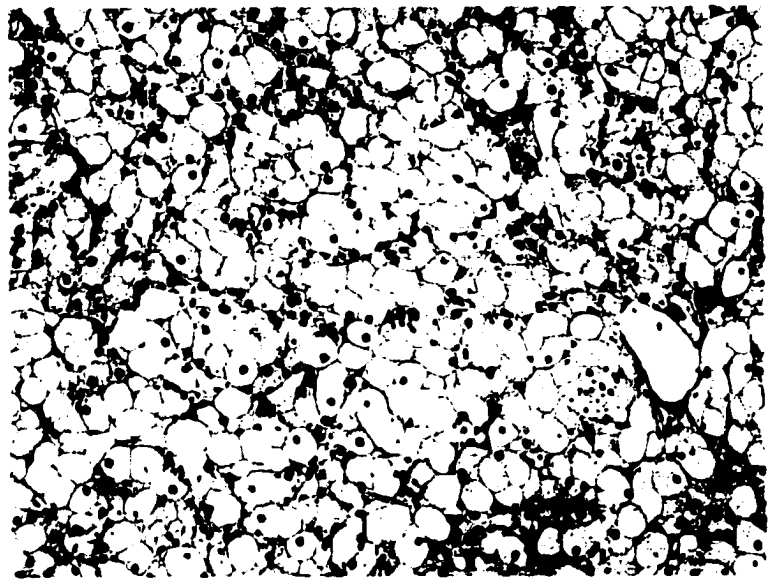


FIGURE 1.1
Liver (H&E 100X)
NPC affected treated cat with discrete microvacuolar pattern in cytoplasm of hepatocytes.

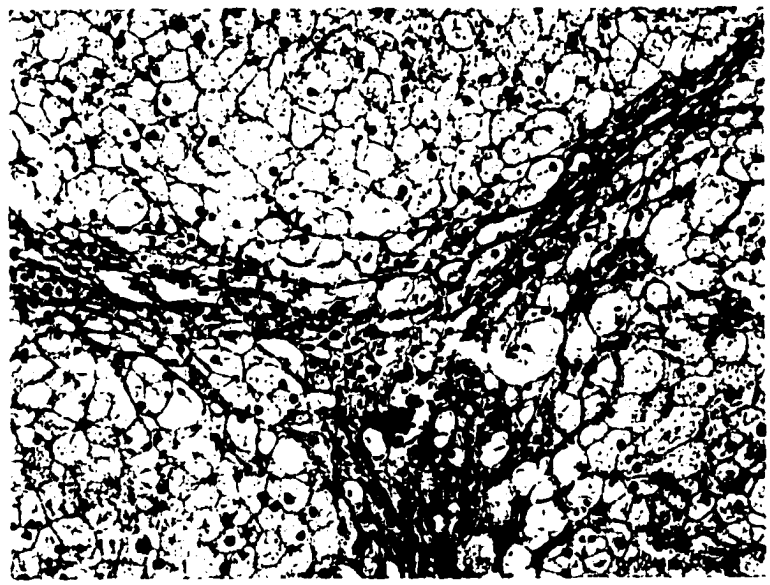


FIGURE 1.2
Liver (H&E 100X)
NPC affected untreated cat with a mixed micro-macro vacuolar pattern in cytoplasm of hepatocytes and prominent fibrosis.

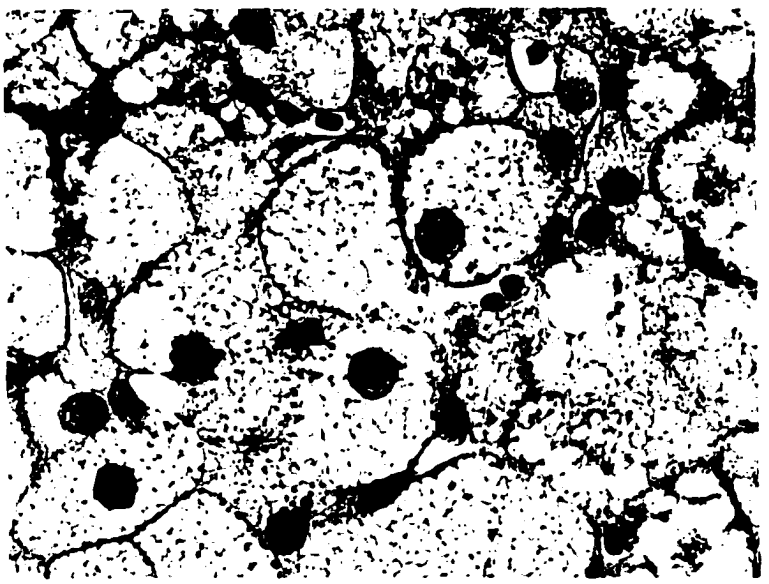


FIGURE 1.3
Liver (H&E 500X)
NPC affected treated cat with discrete microvacuolar pattern in cytoplasm of hepatocytes.

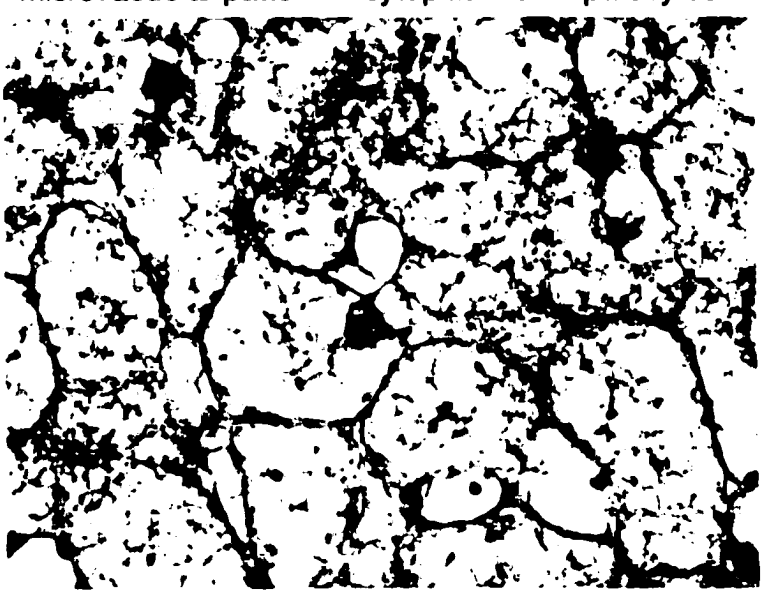


FIGURE 1.4
Liver (H&E 500X)
NPC affected untreated cat with a mixed micro-macrovacuolar pattern in cytoplasm of heptocytes.

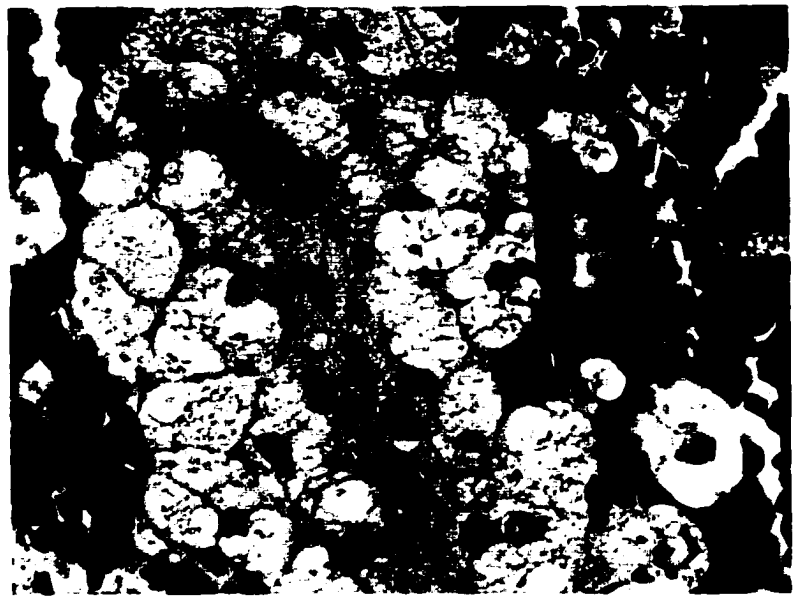


FIGURE 1.5
Spleen (H&E 100X)
NPC affected treated cat with a micro- vacuolar pattern within macrophages.

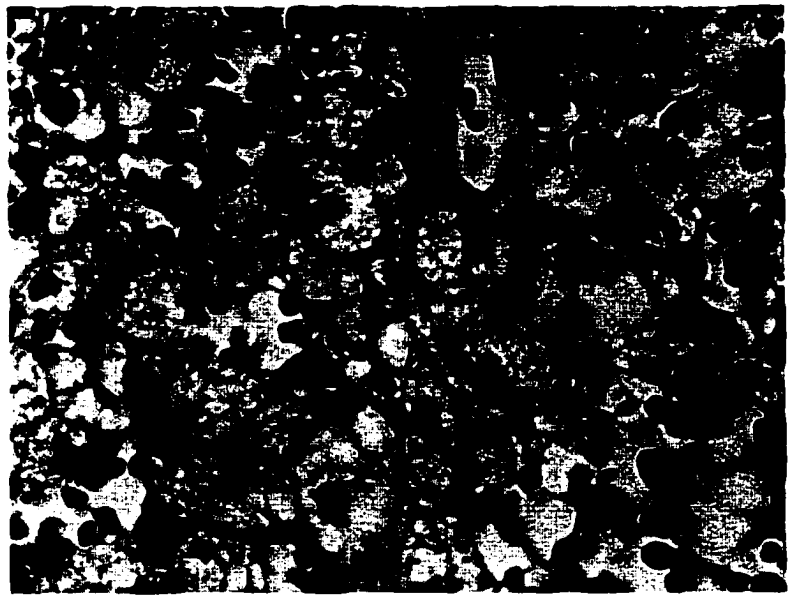


FIGURE 1.6
Spleen (H&E 100X)
NPC affected untreated cat with a mixed micro-macro vacuolar pattern within macrophages.

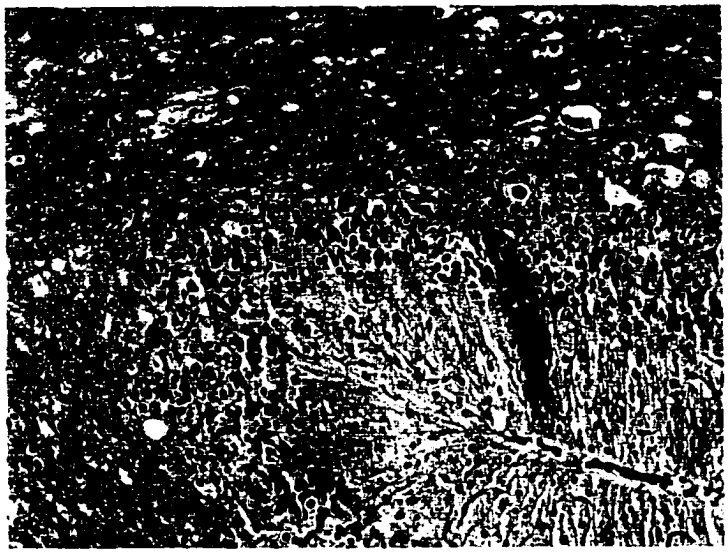


FIGURE 1.7
Cerebellum (H&E 100X)
NPC affected untreated cat with axonal spheroids and Purkinje cell loss.

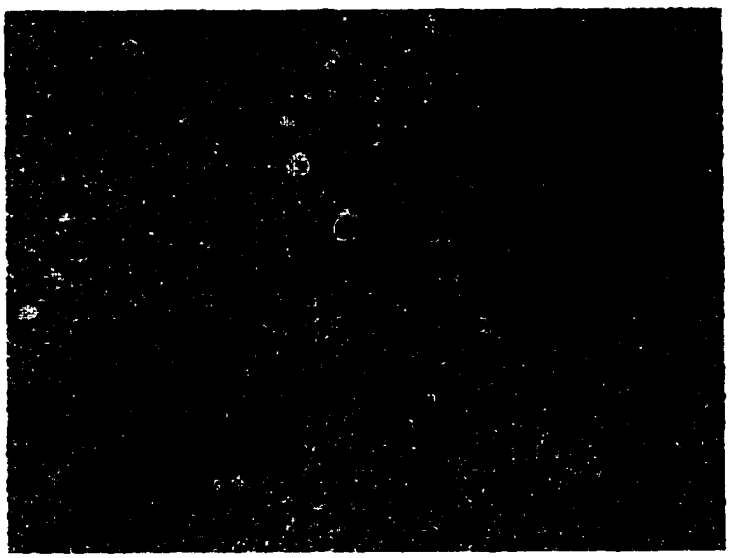


FIGURE 1.8
Cerebellum (H&E 100X)
NPC affected untreated cat with axonal spheroids and Purkinje cell loss.

CHAPTER TWO

EFFECT OF N-BUTYLDEOXYNOJIRIMYCIN IN A FELINE MODEL OF NIEMANN-PICK DISEASE TYPE C

INTRODUCTION

Niemann-Pick Disease Type C (NPC) is an inherited storage disorder characterized by a cholesterol metabolism defect that results in lysosomal accumulation of several types of lipid. The predominant storage material outside the central nervous system (CNS) includes unesterified cholesterol, phospholipids and sphingomyelin (1, 2), in contrast to the storage seen within the CNS, which is predominantly glycosphingolipids (GSL) and in particular, gangliosides (GM2 and GM3) (3, 4). Ganglioside accumulation has been associated with ectopic dendritogenesis in NPC, as well as in several other storage diseases, and may be a contributing factor to the neurological deterioration (3).

The primary goal of this pilot study was to evaluate the impact of the ganglioside synthesis inhibitor, N-butyldeoxynojirimycin (NB-DNJ), on clinical disease progression

and brain ganglioside accumulation in NPC affected cats. N-butyldeoxynojirimycin is an imino sugar that inhibits the ceramide-specific glucosyltransferase that catalyzes the first step of GSL biosynthesis (5, 6). The rationale for this study was that NB-DNJ has been used to successfully prevent the accumulation of GM2 within the brain of Tay-Sachs and Sandhoff mice, in which GM2 is stored (7). Additionally, the Sandhoff mice showed delayed symptom onset, reduced storage in peripheral tissues and increased life expectancy. These findings suggested that NB-DNJ might have a beneficial effect on GSL accumulation in the treatment of NPC and associated neurological dysfunction. NB-DNJ is a potent inhibitor of glycosphingolipid synthesis.

NB-DNJ inhibits the N-linked oligosaccharide processing enzymes α-glucosidases I and II, and the ceramide specific glucosyltransferase which catalyses the first step in glucosphingolipid biosynthesis. The drug is water-soluble, is not metabolized and is excreted intact by the kidney. The hypothesis for this study was that administration of NB-DNJ to NPC affected cats would prevent and/or slow clinical disease progression and decrease brain ganglioside accumulation in the feline model of NPC.

This NB-DNJ drug study was a collaborative effort with Drs. Stephen Walkley and Mark Zervas (Albert Einstein College of Medicine, Bronx, NY) who conducted a concurrent

drug study in NPC mice to evaluate the effect of NB-DNJ on clinical disease progression and ganglioside storage.

MATERIALS AND METHODS

Animals

Cats were divided into four groups that consisted of NPC affected treated cats (n=3), NPC affected untreated cats (n=12, includes historical data), normal treated cats (n=4), and normal untreated cats (established reference ranges). Cats were housed and cared for according to the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, under the guidelines of the Institutional Animal Care and Use Committee (ACUC), and housed in an American Association for Accreditation of Laboratory Animal Care (AAALAC) certified facility at Colorado State University.

NB-DNJ was donated by Oxford GlycoSciences (Abingdon, United Kingdom). The drug was administered as a powder mixed with cat food, or if cats would not eat the food, it was mixed with a small amount of water and administered orally using a syringe. An initial dose of 1200-mg/kg body weight divided bid was given, based on the dosage used in mice. This dose was hepatotoxic; increased liver enzyme activities were markedly increased within 24 hours of administration. The dose was immediately decreased to

150 mg/kg body weight divided bid and later to 50 - 100 mg/kg body weight divided bid, depending on appetite, and presence of weight loss, vomiting, or diarrhea. Table 2.1 summarizes the individual cat status, age at start of study and number of days on treatment for each NB-DNJ dosage.

Clinical Neurological Assessment

Neurological examinations of NPC affected cats were conducted on a bi-weekly basis beginning at 6 weeks of age and continuing until the end of the study. Evaluation of postural, reflex and cranial nerve function was performed by a board certified veterinary neurologist unaware of the treatment status of individual cats. Additionally, all cats were monitored on a daily basis for changes in clinical behavior and eating habits. Normal controls were not neurologically examined.

Hematology and Serum Biochemical Analyses

Complete blood counts and serum biochemical profiles were performed weekly (or more frequently if indicated) and began one week prior to the first administration of the drug. Blood was collected by jugular venipuncture following a 12 hour fast and prior to the morning administration of the drug. Serum analyses were performed on all NPC affected treated and NPC untreated cats and treated normal controls. Serum chemistry analyses were performed on an automated analyzer (Hitachi 704 or 911; Boehringer-

Mannheim Corporation, Indianapolis, IN, USA). Analytes included serum cholesterol, bile acids and bilirubin concentrations, and serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activities. Serum for measurement of NB-DNJ concentration was collected and frozen at multiple time points throughout the study. All serum samples were batch analyzed by Oxford GlycoSciences (Abingdon, United Kingdom).

Brain Ganglioside Analyses

Animals were pre-medicated with a combination of butorphanol, acepromazine and glycopyrolate (BAG protocol) and deeply anesthetized with isofluorane. Sections of cerebral cortex were collected and frozen at –70C; the CNS was immediately perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer followed by immersion fixation in the same fixative for 4 hours.

High Performance Thin Layer Chromatography (HPTLC)

Samples for HPTLC were collected from the left hemisphere from the midline to the lateral extent of cortex in the coronal plane at midline along the anterior posterior axis. Frozen brain tissue was homogenized, then total lipids were extracted by the method of Folch (8). A volume of total lipid extract equivalent to 10 mg dry weight was partitioned. The upper phase was purified by SEP PAK C18 chromatography and dried under N2,

re-suspended, and the extract spotted on silica gel 60-coated glass-backed, HPTLC plates. After drying, plates were developed in cholorform:methanol:0.22% CaCl2 (55:45:10). To image gangliosides, the plate was sprayed with a fine mist of resorcinol and incubated at 100C for 35 minutes until a purple color reaction occurred. Lipids were identified by comparison with authentic ganglioside standards.

Immunohistochemistry (IHC)

Samples for IHC were collected from the right hemisphere from the midline to the lateral extent of cortex in the coronal plane at midline along the anterior posterior axis. Fixed brain tissue sections were processed for immunocytochemistry. Antibody to GM2 ganglioside was employed to observe ganglioside concentration in treated versus untreated cats in a qualitative manner. The cerebral cortex, and the Purkinje cells, and granule cells within the cerebellum were evaluated. Sections for immunocytochemistry were reacted free floating in wells containing the appropriate reagents. Sections were pre-treated with 0.5% hydrogen peroxide in methanol to inactivate endogenous peroxidases, washed in phosphate buffered saline (PBS) , then pre-incubated for one hour in either 10% normal goat or horse serum to block nonspecific antibody binding. Sections were incubated overnight with antibody against GM2 ganglioside (1:1000 dilution, P. Livingston, Memorial Sloan Kettering Medical Center). After an 18 hour incubation at 4C, tissue sections were washed in 0.01 M PBS, incubated for one hour at

room temperature with a biotinylated antibody against the host species of the primary antibody, washed again, and incubated for 1 hour with the avidin-horseradish peroxidase reagent kit (Vectastain ABC kit, Vector, Laboratories). Peroxidase label was demonstrated using a 0.05% 3,3' diaminobenzidine/0.01% hydrogen peroxide mixture diluted in 0.05 M TRIS-HCL pH 7.4. Sections were mounted on gel-coated slides, dehydrated in graded ethanol, and cover slipped in permount.

Morphological Analyses

Tissues for light microscopy were collected at necropsy, fixed in 10% neutral buffered formalin, processed routinely, sectioned at 6μm, and stained with hematoxylin and eosin (H&E). Liver, spleen, adrenal gland, lymph node, kidney, lung, myocardium, thyroid, intestine, and ovaries were examined.

RESULTS

Clinical Neurological Examinations

Progression of clinical neurological disease in the two older NPC affected treated cats was delayed compared to that of NPC affected untreated cats. Onset of clinical signs appeared to be delayed in the youngest NPC affected treated cat compared to NPC affected untreated controls. Cats placed on the drug treatment after the onset of clinical signs had less severe intention tremors compared to age-matched NPC untreated cats.

Additionally, the cat that began treatment at 21 weeks became ataxic at a much later time point compared to NPC affected untreated cats. The NPC affected treated cat that began treatment at 13 weeks of age died at 21 weeks of age (from pneumonia) and had no evidence of ataxia.

The cat that began the drug treatment at 7 weeks of age, prior to the onset of clinical signs, was clinically normal at 11 weeks, the age of death. This is in contrast to NPC affected untreated cats that typically show signs of subtle head intention tremors by 10 weeks of age (n=12), although onset as late as 12 weeks (2 out of 12 kittens or 17%) has been noted. This cat had severe hypoglycemia that was likely the cause of death and possibly attributable to the drug treatment. The age of onset for clinical neurological signs for each cat / group is summarized in Table 2.2. Values given for NPC affected untreated cats (AUTC) are group means (n=12) and include historical data.

Hematology, Serum Biochemical Analyses and Serum Drug Concentrations

Abnormalities seen in the complete blood counts included lymphocytosis, likely attributable to an excitement response that is common in young cats. At different time points, transient neutrophilia with low to normal lymphocyte concentration was present; this combination is most consistent with an endogenous steroid induced stress response.

Mild hypoproteinemia characterized by hypoglobulinemia was seen in all groups and likely reflects the limited antigenic exposure of cats in a closed research colony; this has been a consistent observation in this particular colony of cats. Hypercholesterolemia of comparable magnitudes was seen in both NPC affected treated and NPC affected untreated cats. Transient hypoglycemia was noted in one NPC affected treated cat and one normal treated cat. Increased serum AST and ALT activities were seen in all groups except the non-treated normal controls, and was exacerbated by increased dosages of NB-DNJ (Figures 2.1, 2.2 and 2.4). Mild increases in serum ALP activity and serum phosphorus were seen in all groups of cats and is most consistent with normal increases seen in young growing animals. Additionally, increases in serum ALP activity in normal treated cats which were greater than increases associated with bone growth were seen as a NB-DNJ dose related response. Serum ALP activity was decreased in NPC affected treated cats relative to NPC affected untreated cats. NPC affected non-treated cats had serum ALP activities that were greater than the age related increases seen in normal young cats (Figures 2.3 and 2.4). Bile acids were mildly increased in all groups except the normal non-treated control cats, and occasional concurrent hyperbilirubinemia was noted. The increases seen in the NPC affected treated and untreated cats were comparable to increases seen historically in all NPC affected cats. The increased bile acids in the normal treated cats appeared to be associated with high drug dosages.

Serum drug concentrations were determined for multiple time points and various drug dosages. At drug dosages of 50-75 mg/kg/day, cats had a mean serum drug concentration of 1916 ng/ml (range 150-5548 ng/ml); at dosages of 100-150 ng/ml serum concentrations had a mean value of 19,255 ng/ml (range 11,316-30,000 ng/ml). Increases in administered drug dosages correlated with increases in serum concentrations of NB-DNJ up to 100 mg/kg/day at which point the serum drug concentration reached a plateau (Figure 2.5).

Brain Ganglioside Analyses

HPTLC

Analysis of cerebral cortex demonstrated qualitatively decreased concentrations of GM3 and GM2 gangliosides in the cerebral cortex of one NPC affected treated cat evaluated to date. Analysis of cerebral cortex of normal treated and normal untreated cats showed that they have undetectable levels of GM3 and GM2 gangliosides compared to NPC affected cats (Figure 2.6).

Immunohistochemistry

Immunocytochemical staining of the cerebral cortex and cerebellum with anti-GM2 antibody showed a qualitative reduction in storage material in the NPC affected treated cat. In the cerebral cortex reduced storage was most apparent in the pyramidal

neurons, while in the cerebellum the reduction was most notable in the granule cell layer (Figures 2.7 and 2.8). In the NPC affected untreated cat, punctate vesicular GM2 labeling was extensive and intensely labeled numerous pyramidal cells of the cerebral cortex. Also, Purkinje cells of the cerebellar cortex and the entire granule cell layer displayed marked GM2 labeling.

Morphological Analyses

Liver

Inflammatory cells were present in the portal and periportal regions of the livers of one NPC affected treated cat and the adult treated normal control cat. The typical NPC related vacuolization of hepatocytes and Kupffer cells was similar in both the NPC affected treated and NPC affected untreated cats.

Adrenal

One treated normal cat and one treated affected cat had moderate to severe adrenal cortical degeneration. Foci of swollen degenerate cells and areas of calcification were present in the zona fasiculata of the adrenal cortex.

Other Tissues

Mild intestinal cryptitis was present in one NPC affected treated cat. No other morphological abnormalities, besides the typical NPC vacuolization, were noted in sections of kidney, lung, lymph node, spleen, myocardium, pancreas or thyroid

evaluated from cats in each of the treatment groups. No differences in NPC related lesions were noted in tissues from NPC affected treated cats compared to tissues from NPC affected untreated cats.

DISCUSSION

Progression of clinical neurological disease in the two older NPC affected treated cats was slower than progression of disease in the NPC affected untreated cats. Onset of clinical signs was possibly delayed in the youngest NPC affected treated cat that was clinically normal at 11 weeks of age, compared to NPC affected untreated cats that typically show clinical signs by 10 weeks of age. Both HPTLC and immunocytochemical staining suggested that ganglioside accumulation in NPC affected treated cats was qualitatively less, compared to the NPC affected untreated cats. These results are similar to those seen in NPC affected mice treated with NB-DNJ (Zervas, Somers et al, submitted to Science 10/2000). However the study duration was limited and the number of treated cats was small.

The qualitative reduction of GM2 and GM3 ganglioside storage within the CNS is significant in light of concurrent clinical neurological improvement, and may implicate ganglioside accumulation as a possible contributing factor to neurological dysfunction in NPC. The apparent reduction in gangliosides based on HPTLC was subjective and may

be a result of sample loading. Quantitative analysis of gangliosides is crucial for definitive interpretation of data and analyses are pending.

The dosage used in mice (1200mg/kg/day) was hepatotoxic in cats based on increased liver enzyme activities (AST, ALT and ALP) and abnormalities in hepatic function indicators (serum bile acid and bilirubin concentrations). These serum abnormalities correlated with increased dosages of NB-DNJ. The cat treated prior to the onset of clinical signs died at an early point in this study and the death was likely due to hypoglycemia. Hypoglycemia may have been related to the drug or possibly a result of anorexia that was also noted in the cat. Decreased liver function can result in hypoglycemia, but no evidence of end stage liver disease was present in the cat. Previous studies using NB-DNJ in mice have documented weight loss and lymphoid depletion. Although weight loss was seen in the treated cats, no lymphoid depletion was identified. However, adrenal cortical necrosis was seen in two treated cats (one normal and one NPC affected). Differences in drug administration to mice compared to cats may account for some of the different types of toxicity observed in the two species. The NB-DNJ was incorporated into dry mouse chow, thus mice consumed the drug over a long period of time whereas cats received bolus doses of the drugs twice daily. Moreover, mice require two times the NB-DNJ dose used in humans to achieve the same serum concentration (7). Additionally, cats may be uniquely sensitive to NB-DNJ

due to species related differences in liver metabolism. Pharmacokinetic studies would be useful for determining ideal ND-DNJ dosages to be used in cats. The administration of a less toxic inhibitors such as N-butyldeoxygalactonojirimycin may avoid some of the negative side affects associated with NB-DNJ (9).

Interestingly, studies using double knockout mice revealed that the absence of ganglioside accumulation had no impact on NPC related neurological signs and that visceral storage remained unchanged. NPC model mice were bred with mice carrying a mutated GalNAcT gene, which is responsible for the synthesis of GM2. No reduction in visceral storage was noted in the double mutants. However, they had significant reduction in neuronal storage and decreased staining of neurons with filipin, but no improvement in the clinical NPC phenotype (10). Demyelination and neurodegeneration as determined by apoptotic cells and loss of Purkinje cells persisted as a prominent feature in the double mutants. These findings raise questions about the true role ganglioside accumulation and the formation of ectopic dendrites as they relate to NPC and the characteristic neurological deterioration.

In addition to ectopic dendritogenesis, a prominent feature in the brain of NPC cats is the formation of axonal spheroids. These are found predominantly on GABAergic cells including the Purkinje cells (11). Axonal spheroid formation is a prelude to neuroaxonal dystrophy and subsequent cell death. The progressive loss of Purkinje cells is strongly

associated with worsening of the clinical NPC phenotype (11). Axonal spheroids are thought to result from a block in retrograde transport (12). Perhaps the NPC1 protein is critical to the retrograde transport of endocytosed material from the distal axons, and possibly formation of axonal spheroids, followed by neuroaxonal dystrophy and eventual cell death is responsible for the NPC phenotype. The NPC1 protein has been demonstrated to be crucial for retrograde transport within the endocytic system and when defective, results in non-specific storage of numerous materials(13-15) .

The preliminary results of this limited pilot study are encouraging and additional studies should be performed. Ganglioside synthesis inhibitors show more promise than any treatment modality evaluated to date; human clinical trials using NB-DNH to treat NPC were initiated in the Fall of 2000. This feline model of NPC is a valuable resource for the continued evaluation of ganglioside synthesis inhibitors and well as other therapeutic modalities.

REFERENCES

1. Pentchev, P. G., Brady, R. O., Blanchette-Mackie, E. J., Vanier, M. T., Carstea, E. D., Parker, C. C., Goldin, E., and Roff, C. F. (1994) The Niemann-Pick C lesion and its relationship to the intracellular distribution and utilization of LDL cholesterol. *Biochim Biophys Acta* 1225, 235-243

2. Pentchev, P. G., Comly, M. E., Kruth, H. S., Vanier, M. T., Wenger, D. A., Patel, S., and Brady, R. O. (1985) A defect in cholesterol esterification in Niemann-Pick disease (type C) patients. *Proc Natl Acad Sci U S A* 82, 8247-8251

3. Walkley, S. U., Zervas, M., and Wiseman, S. (2000) Gangliosides as Modulators of Dendritogenesis in Normal and Storage Disease-affected Pyramidal Neurons. *Cereb Cortex* 10, 1028-1037

4. Watanabe, Y., Akaboshi, S., Ishida, G., Takeshima, T., Yano, T., Taniguchi, M., Ohno, K., and Nakashima, K. (1998) Increased levels of GM2 ganglioside in fibroblasts from a patient with juvenile Niemann-Pick disease type C. *Brain Dev* 20, 95-97

5. Platt, F. M., Neises, G. R., Dwek, R. A., and Butters, T. D. (1994) N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. *J Biol Chem* 269, 8362-8365

6. Platt, F. M., Neises, G. R., Karlsson, G. B., Dwek, R. A., and Butters, T. D. (1994) N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing. *J Biol Chem* 269, 27108-27114

7. Platt, F. M., Neises, G. R., Reinkensmeier, G., Townsend, M. J., Perry, V. H., Proia, R. L., Winchester, B., Dwek, R. A., and Butters, T. D. (1997) Prevention of lysosomal storage in Tay-Sachs mice treated with N- butyldeoxynojirimycin. *Science* 276, 428-431

8. Folch, J., Lees, M, Sloane Stanley GH (1957) A simple mthod for the isolation and purification of total lipids from animal tissues. *Journal of Boilogical Chemistry*, 497-509

9. Andersson, U., Butters, T. D., Dwek, R. A., and Platt, F. M. (2000) N-butyldeoxygalactonojirimycin: a more selective inhibitor of glycosphingolipid biosynthesis than N-butyldeoxynojirimycin, in vitro and in vivo. *Biochem Pharmacol* 59, 821-829

10. Liu, Y., Wu, Y. P., Wada, R., Neufeld, E. B., Mullin, K. A., Howard, A. C., Pentchev, P. G., Vanier, M. T., Suzuki, K., and Proia, R. L. (2000) Alleviation of

neuronal ganglioside storage does not improve the clinical course of the Niemann-Pick C disease mouse. *Hum Mol Genet* 9, 1087-1092

11. March, P. A., Thrall, M. A., Brown, D. E., Mitchell, T. W., Lowenthal, A. C., and Walkley, S. U. (1997) GABAergic neuroaxonal dystrophy and other cytopathological alterations in feline Niemann-Pick disease type C. *Acta Neuropathol (Berl)* 94, 164-172

12. Walkley, S. U. (1998) Cellular pathology of lysosomal storage disorders. *Brain Pathol* 8, 175-193

13. Neufeld, E. B., Wastney, M., Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Roff, C. F., Ohno, K., Morris, J. A., Carstea, E. D., Incardona, J. P., Strauss, J. F., 3rd, Vanier, M. T., Patterson, M. C., Brady, R. O., Pentchev, P. G., and Blanchette-Mackie, E. J. (1999) The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J Biol Chem* 274, 9627-9635

14. Higgins, M. E., Davies, J. P., Chen, F. W., and Ioannou, Y. A. (1999) Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol Genet Metab* 68, 1-13

15. Watari, H., Blanchette-Mackie, E. J., Dwyer, N. K., Glick, J. M., Patel, S., Neufeld, E. B., Brady, R. O., Pentchev, P. G., and Strauss, J. F., 3rd (1999)

Niemann-Pick C1 protein: obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. *Proc Natl Acad Sci U S A* 96, 805-810

Table 2.1

Age at Study Start and Number of Day on Each Drug Dosage

Cat ID	NPC Status	Age in weeks at study start	Days on 1200 mg/kg/day	Days on 150 mg/kg/day	Days on 100 mg/kg/day	Days on 50 mg/kg/day	Total days on Drug
161	Normal	136	0	0	11	8	19
218	Normal	21	1	20	23	11	54
235	Normal	13	0	0	0	144	144
232	Normal	7	0	0	9	14	23
222	Affected	21	1	20	23	11	54
231	Affected	13	0	0	0	156	156
233	Affected	7	0	0	9	14	23

Table 2.2

Age in weeks for onset of neurological signs

Cat ID	Treatment Status	Age at Study Start	Intention Tremors	Ataxia	Falling	Age at Death
222	treated	21	11	29	none	33
231	treated	13	6	none	none	20
233	treated	7	none	none	none	11
AUTC	untreated	N/A	10	14.5	24.5	33

AUTC – affected untreated controls (n=12)

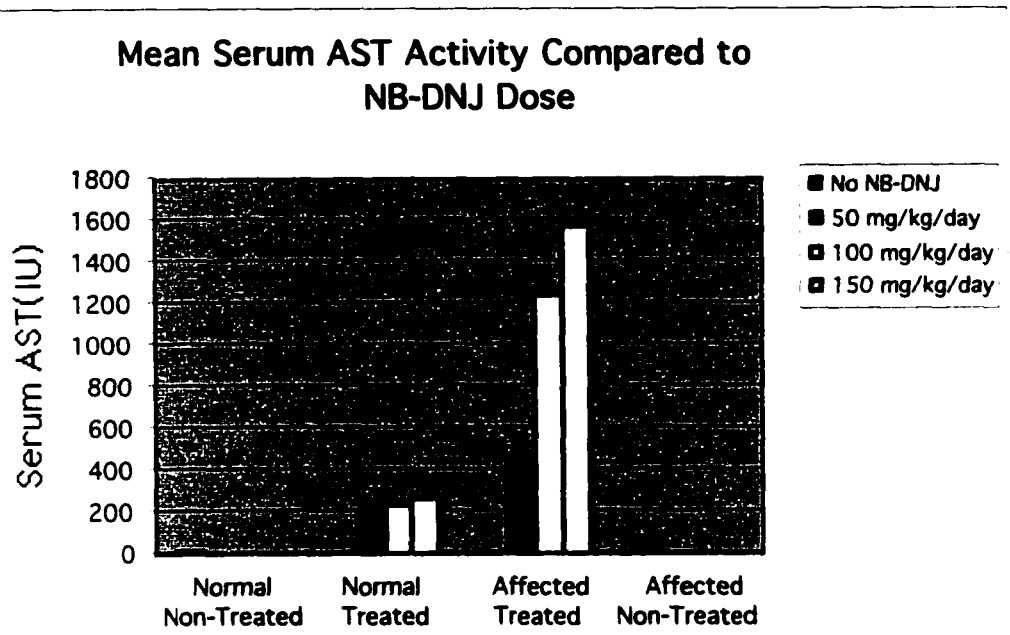


FIGURE 2.1

Serum AST increased as the dosage of NB-DNJ increased; the magnitude of this increase was greater in NPC affected treated cats compared to that of normal treated cats.

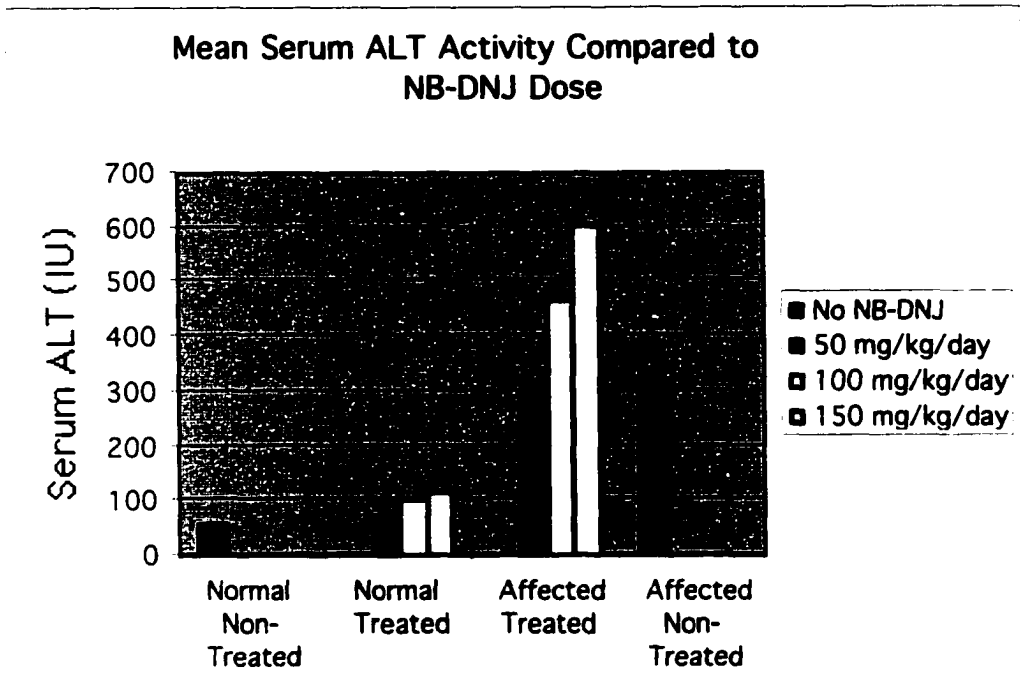


FIGURE 2.2

Serum ALT activity increased as the dosage of NB-DNJ increased; the magnitude of increase was greater in NPC affected treated cats compared to normal treated cats. ALT activity in NPC affected non-treated cats was also increased.

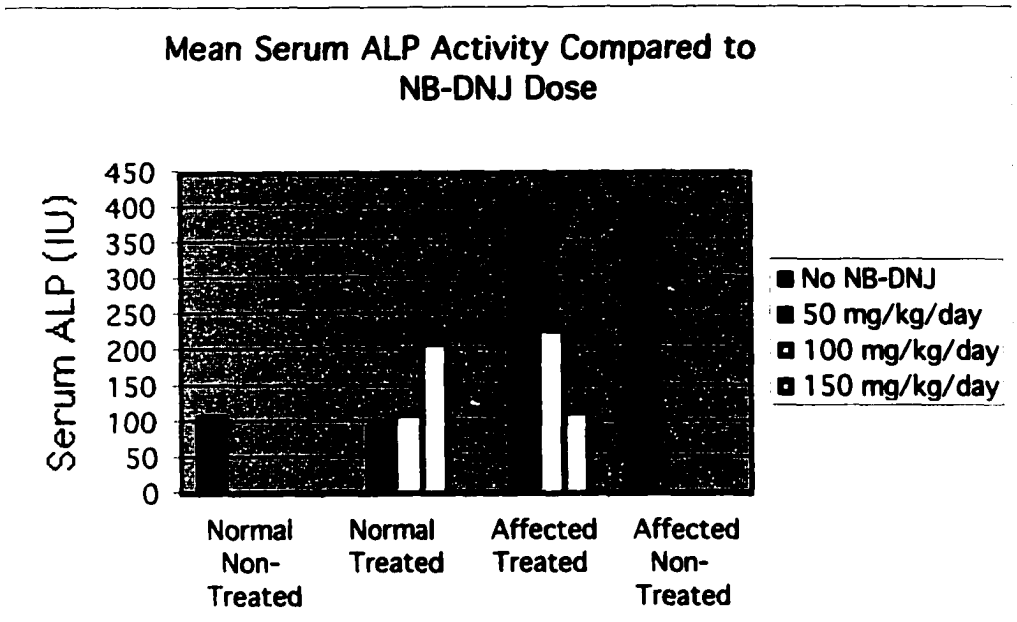


FIGURE 2.3

Serum ALP activity increased in normal treated cats as the dosage of NB-DNJ increased while serum ALP activity decreased in NPC affected treated cats as the NB-DNJ dose increased. ALP activity in NPC affected non-treated cats was also increased.

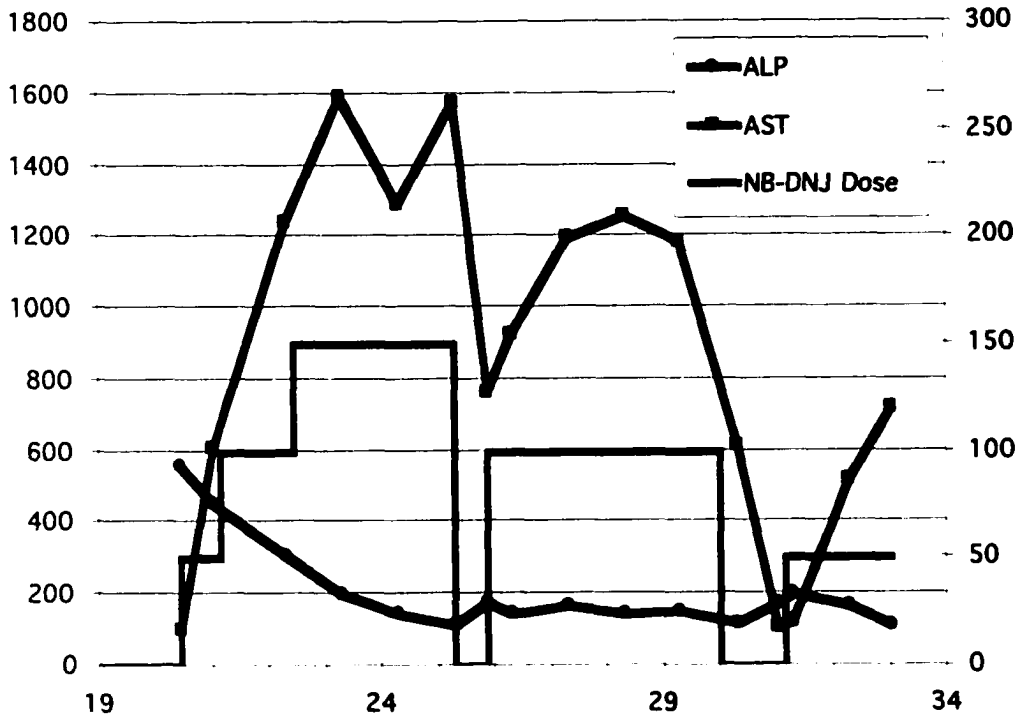


Figure 2. 4

NB-DNJ Dose Versus Serum ALP and AST Activity

Data from one NPC affected treated cat.

Serum ALP and AST (IU, y-axis)
Serum NB-DNJ on the right (ng/ml)

As the serum NB-DNJ concentration increased, serum ALP activity decreased and serum AST activity increased.

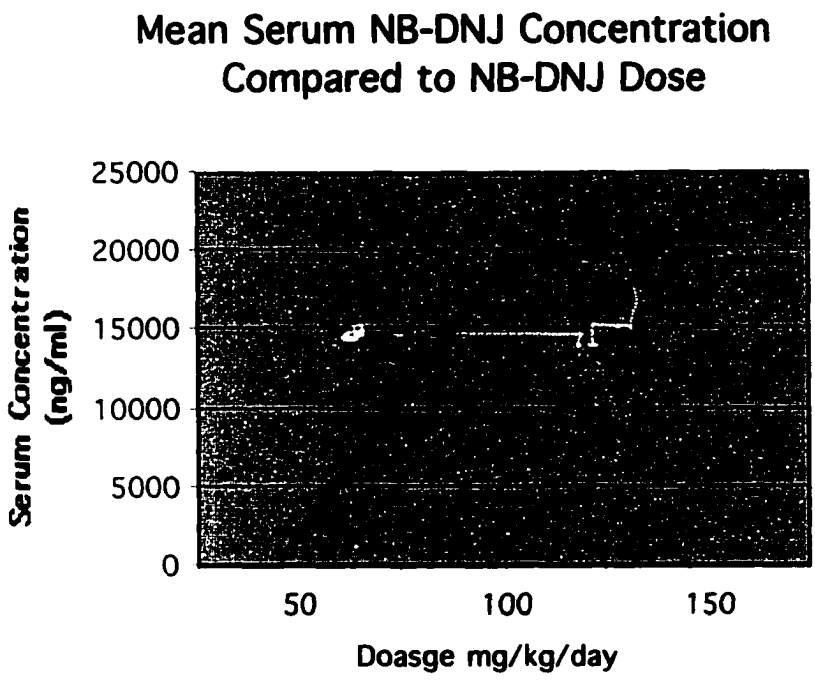


FIGURE 2.5

Serum concentration of NB-DNJ increased as the drug dosage was increased up to approximately 100/mg/kg at which point serum concentrations reached a plateau of ~20,000 ng.ml.

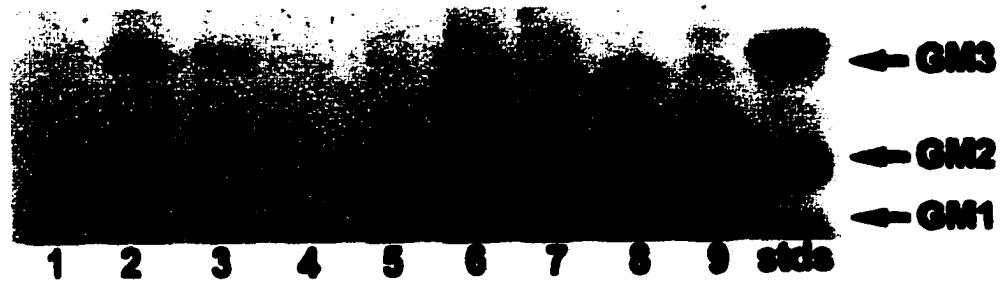


Figure 2.6

HPTLC Plate - Ganglioside Content of Cerebral Cortex

Lane 1 - Normal treated cat
Lane 2 - NPC affected non-treated cat
Lane 3 - NPC affected treated
Lane - 4 Normal non-treated cat

Lane 3 shows decreased GM3 and GM2 content compared to lane 4.

Lanes 5-9 correspond to murine samples from a concurrent study

Lane 5 - Normal non-treated mouse
Lane 6 - NPC affected non-treated mouse
Lanes 7 and 8 - NPC affected treated mice
Lane 8 - Normal treated mouse

Lanes 7 and 8 show decreased GM3 and GM2 content compared to lane 6.

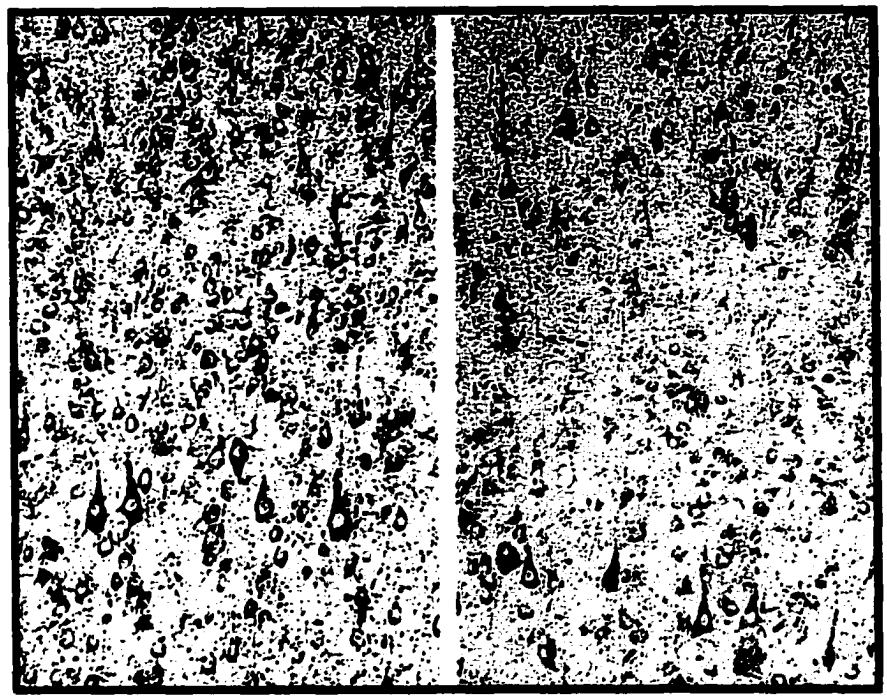


Figure 2.7

Immunocytochemistry of Anti-GM2 Reactivity in Cerebral Cortex

Left - NPC affected non-treated cat
Right - NPC affected treated cat

GM2 immunoreactivity is less apparent in the NPC affected treated cat on the right.

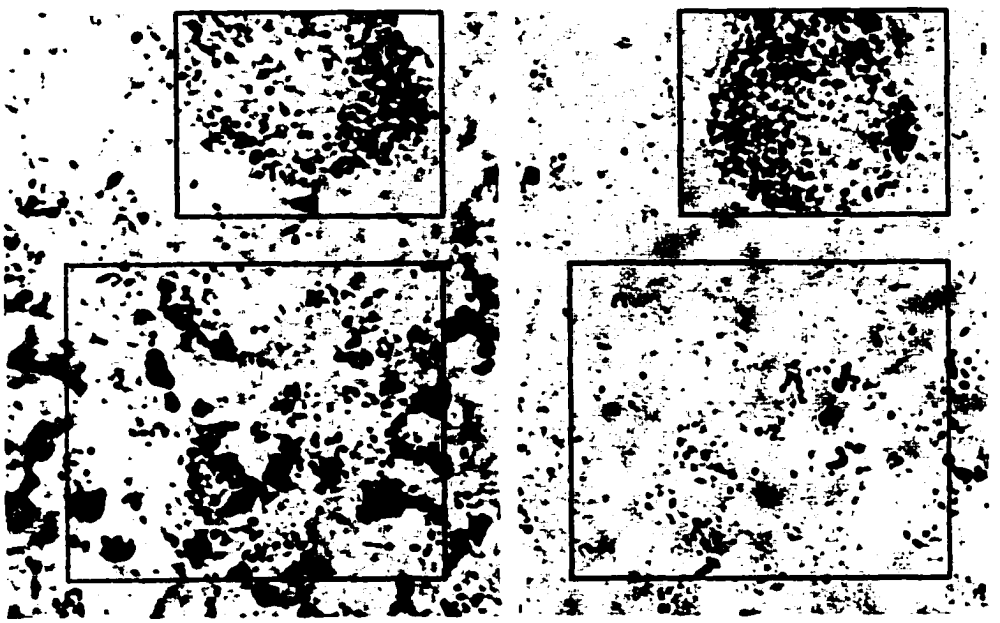


Figure 2.8

GM2 Immunoreactivity in the Cerebellum

Left side represents an NPC affected non-treated cat, which displays more anti-GM2 reactivity than is seen in the NPC affected NB-DNJ treated cat on the right.

The decreased immunoreactivity is more apparent in the granule cell layer (red box) than in the Purkinje cells (black box)

CHAPTER THREE

COMPLEMENTATION STUDIES IN HUMAN AND FELINE NIEMANN-PICK DISEASE TYPE C

INTRODUCTION

Complementation studies involve restoration of normal cellular function after the hybridization of two different cell lines. Normal cellular function can be restored when a competent cell line provides the missing genetic information that is responsible for the production of the absent or defective gene product. When two cell lines are crossed and no restoration of normal cellular function occurs, the mutation in each cell line involves the homologous gene. Complementation studies can be used to identify diseases in which mutation of two separate genes result in a common phenotype that is otherwise indistinguishable. This has been demonstrated in human Niemann-Pick Disease Type C (NPC) (1,2,3). Additionally, complementation studies have been used to confirm that animal models that appear to represent a human disease have the same genetic basis (4,5). Complementation studies were used to identify a murine model of

NPC that belongs to the same complementation group as the major form of childhood NPC (6).

The feline model of NPC has been shown to be clinically, biochemically, and morphologically identical to the major form of human NPC, NPC1 (7,8,9,10). Niemann-Pick Disease Type C is characterized by the abnormal accumulation of unesterified cholesterol in lysosomes that can be visualized by staining cultured fibroblasts with the fluorescent antibiotic filipin, which specifically binds unesterified cholesterol.

In this study, complementation experiments using cultured fibroblasts from NPC affected cats and NPC-1 affected humans were conducted to determine if this feline model involves the gene orthologous to the human gene, *NPC1*. Cell fusions between human NPC and feline NPC fibroblasts were conducted to assess whether the multinucleated heterokaryons that were formed showed a reversal of the NPC phenotype.

Specific Aims - Complementation studies were performed to determine if the gene responsible for the major form of human Niemann-Pick type C disease (NPC) and the feline model of NPC are orthologous.

Hypothesis – Cell fusions between feline NPC fibroblasts and human NPC fibroblasts will result in the formation of multinucleated heterokaryons without the restoration of

normal cellular function, and this lack of complementation can be visualized by the retention of normal NPC phenotype as seen with filipin staining.

MATERIALS AND METHODS

Fibroblast strains and cell fusion

The following cell lines were evaluated: human NPC1 affected fibroblasts (two unrelated patients), feline NPC affected fibroblasts (three cats from the NPC colony), and feline normal fibroblasts (two cats from outside the NPC colony). Crosses performed are summarized in Table 1. Fibroblasts were cultured using standard protocols, and cell fusions were performed according to the methods of Brul et al (11). Fibroblasts were cultured in 25cm^2 flasks in regular media (Minimal Essential Media (MEM) supplemented with 10% fetal calf serum (FCS), 2% L-glutamine (Irvine Scientific) and gentamycin). Cell strains from confluent flasks were mixed and co-cultured in a ratio of 1:1 for three days. Cell fusions were performed on day 4 according to the following protocol. Media was removed from flasks of co-cultured fibroblasts and cells were rinsed with MEM; 0.6 ml of 41% (vol/vol) polyethylene glycol 1000 (PEG 1000, Sigma) in MEM with 8.75% (vol/vol) dimethyl sulfoxide (DMSO) was then added. After 2 minutes of rocking, 0.6ml of 25% PEG in MEM was added followed by another 2 minutes of rocking. The PEG suspension was diluted two more times at 2-minute intervals with the addition of 4 mls of

MEM at each time point. The final suspension was aspirated and the cells were rinsed with MEM. The hybrids were then cultured for 3 days in regular media.

Assessment of Complementation

Following fusion, fibroblasts were cultured for three days in MEM supplemented with 10% FCS, 2% L-glutamine and gentamycin under 5% CO_2/95% air. On day four, the media was changed to 5% lipoprotein deficient media (LPDS, Bionetics). Cells were cultured for another 48 hours and then transferred to Lab Tek Chamber slides (NUNC) for an additional 48 hours. Cells were challenged with low density lipoprotein (LDL, Bionetics) at a concentration of 50ug/ml for 24 hours, then fixed with 3% paraformaldehyde and stained with the fluorescent antibiotic filipin. Slides were viewed by fluorescent microscopy, and multinucleated cells were evaluated for complementation.

Verification of Interspecies Fusions

To verify that interspecific fusions had occurred, fibroblasts from human and feline cell lines were differentially labeled with fluorescent cell tracking dyes prior to fusion. Cell tracking dyes used for this study were 5(and 6)-(((4-chloromethyl) benzoyl) amino) tetramethyl rhodamine (CMTMR; Molecular Probes), which appears red, and 5-

chloromethylfluorescein diacetate (CMFDA; Molecular Probes), which appears green. The dyes, as supplied by the manufacturer, were brought up in DMSO to a stock concentration of 5mM. Stock solutions were stored at 4°C in 100μl aliquots. For staining, the dyes were further diluted to 10μM by adding 4μl of 5mM stock dye to 2mls 1X PBS (phosphate buffered saline). Human cells were stained in 10μM CMTMR (red) and feline cells were stained in 10μM CMFDA (green) for 30 minutes at 37°C. Dyed cells were co-cultured and fused by the preceding protocol, then viewed by dual fluorescence microscopy to evaluate multinucleated cells for interspecific fusion. Multinucleated heterokaryons resulting from interspecific fusion would be expected to appear yellow.

RESULTS

Complementation Analysis

All of the visualized multinucleated cells derived from each of the crosses between human NPC affected fibroblasts and feline NPC affected fibroblasts retained the NPC staining phenotype characterized by a prominent perinuclear fluorescence with filipin, indicative of a lack of complementation (Figure 1). Multinucleated cells formed between different feline NPC affected cells all retained the NPC phenotype. Crosses performed between normal feline fibroblasts and NPC affected human or NPC affected feline fibroblasts each resulted in multinucleated heterokaryons that exhibited loss of the NPC

staining phenotype as visualized by the absence of perinuclear fluorescence with filipin, indicating restoration of normal cellular function. (Figure 2).

Interspecific Fusions

Fusions between CMTMR stained human cells (red) and CMFDA stained feline cells (green) resulted in the formation of multinucleated cells with dual fluorescence, which appears yellow (Figure 3).

DISCUSSION

Complementation studies are based upon restoration of normal cellular functions as a result of hybridization of a defective cell with a competent cell. Our goal was to determine if the gene responsible for the NPC phenotype in the feline model of NPC is orthologous to the gene that is mutated in the major group of human NPC, NPC-1. The absence of complementation in all visualized multinucleated cells formed between the fusion of human NPC affected fibroblasts and feline NPC affected fibroblasts suggests that this feline model of NPC and major form of human NPC, NPC-1 arise from mutations of orthologous genes. Attempts to simultaneously filipin stain and track fibroblasts with the fluorescent dyes were not successful, as the tracking dyes did not survive the filipin staining protocol. However, in the verification of interspecific fusion, the

multinucleated heterokaryons that were identified as human/feline hybrids represented greater than 75% of all the multinucleated cells present. Considering this, the retention of the NPC phenotype in every multinucleated cell seen after the fusion of NPC1 affected human and NPC affected feline fibroblasts is significant. Thus, normal cellular function was not restored after hybridization of NPC human and NPC feline fibroblasts, indicating a lack of complementation. Based on these findings, this feline colony appears to have a mutation of the gene orthologous to that responsible for the major form of human NPC1 and will serve as a valuable model for future studies, including evaluating efficacy of gene therapy.

REFERENCES

1. Vanier MT, Duthel S, Rodgriguez-Lafrasse C, Pentchev P, Carstea ED. Genetic Heterogeneity in Niemann-Pick C Disease: A Study Using Somatic Cell Hybridization and Linkage Analysis. *Am J Hum Genet* 58:118-125, 1996.

2. Steinberg SJ, Ward CP, Fensom, AH. Complementation Studies in Niemann-Pick Disease Type C Indicate the Existence of a Second Group. *J Med Gen* 31:317-320, 1994.

3. Dahl NK, Daunais MA, Liscum L. A Second Complementation Class of Cholesterol Mutants with a Variant Niemann-Pick Type C Phenotype. *J Lip Res* 35:1839-1849, 1994.

4. Ahern-Rindell A, Murnane R, Prieur D. Interspecific Genetic Complementation Analysis of Human and Sheep Fibroblasts with B-Galactosidase Deficiency. *Som Cell Mol Gen* 15:525-533, 1989.

5. Hu P, Wenger D, van Diggelen O, Kleijer W. Complementation Studies in Human and Caprine B-Mannosidosis. *J Vet Inter Med* 14:13-17, 1991.

6. Akaboshi S, Yano T, Miyawaki S. A C57BL/KsJ Mouse Model of Niemann-Pick Disease (SPM) Belongs to the Same Complemenation Group as the Major

Childhood Type of Niemann-Pick Disease Type C. *Hum Genet* 99:350-353, 1997.

7. Brown DE, Thrall MA, Walkley SU, Wenger DA, Mitchell TW, Smith MO, Royals KL, March, PA, Allison RW. Feline Niemann-Pick Disease Type C. *AJP* 144:1412-1415, 1994.

8. Brown DE, Thrall MA, Walkley SU, Wenger DA, Mitchell TW, Allison RW, Smith MO, Royals KL, March PA. Animal Model of Human Disease: Feline Niemann-Pick Disease Type C. *Comparative Pathology Bulletin* Vol XXVI 4-6, Aug. 1994.

9. Brown DE, Thrall MA, Walkley SU, Wurzelmann S, Wenger DA, Allison RW, Just CA. Metabolic abnormalities in feline Niemann-Pick type C heterozygotes. *J Inher Metab Dis* 19:1-12, 1996.

10. Muñana KR, Luttgen PJ, Thrall MA, Mitchell TW, Wenger DA. Neurological Manifestations of Niemann-Pick Disease Type C in Cats. *J Vet Inter Med* 8:117-121, 1994.

11. Brul S, Westerveld A, Strijland A, Wanders JA, Schram AW, Heymans HAS, Schutgens RBH, van den Bosch H, Tager JM. Genetic heterogeneity in the cerebrohepatorenal (Zellweger) syndrome and other inherited disorders with a generalized impairment of peroxisomal functions. *J Clin Invest* 81:1710-1715, 1988.

Table 1

Crosses performed for complementation analyses

Human		Feline	Feline		Feline
NPC-H1	X	NPC-F1	NPC-F1	X	NPC-F2
		NPC-F2			NPC-F3
		NPC-F3			NOR-F
		NOR-F	NPC-F2	X	NOR-F3
NPC-H2	X	NPC-F1			NOR-F
		NPC-F2	NPC-F3	X	NOR-F
		NPC-F3			
		NOR-F			

NPC-H1, NPC-H2 represent NPC affected human fibroblast strains

NPC-F1, NPC-F2, NPC-F3 represent NPC affected feline fibroblast strains

NOR-F represents normal feline fibroblast strain

Figure 3.1

The two mononuclear fibroblasts exhibit the perinuclear fluorescence that is typical of the NPC phenotype. The multinucleated cell was formed following the fusion of NPC affected human and NPC affected feline fibroblasts. The persistent presence of marked perinuclear fluorescence is indicative of a lack of complementation. (filipin, 400X)

Figure 3.2

Multinucleated fibroblast formed after fusion of NPC affected human fibroblasts and normal feline fibroblasts. Note the absence of the perinuclear fluorescence in the multinucleated cell, indicative of complementation and restoration of normal cellular phenotype. Human mononuclear cells retained the NPC staining phenotype. (filipin,, 400X)

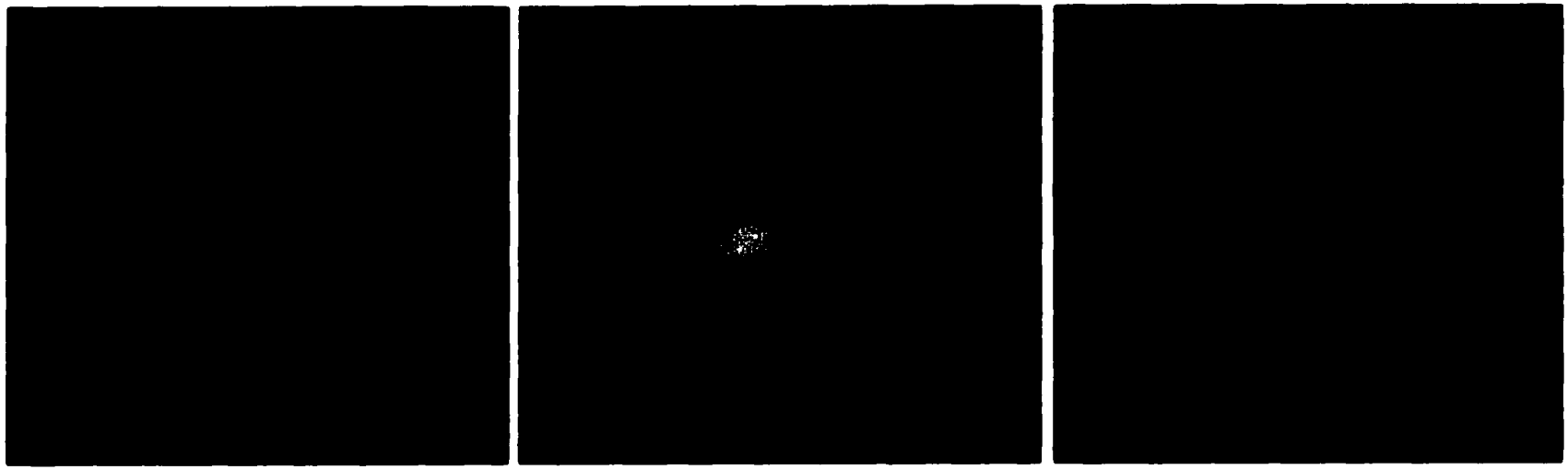


Figure 3.3

(A) Feline NPC affected fibroblasts stained with CMFDA. (C) Human NPC affected fibroblasts stained with CMTMR. (B) Multinucleated heterokaryon as indicated by the dual (yellow) fluorescence as a result of fusion between human and feline cells. (1000X)

CHAPTER FOUR

GENETIC CHARACTERIZATION OF FELINE NIEMANN-PICK DISEASE TYPE C

INTRODUCTION

Niemann-Pick Disease Type C (NPC) was first described over thirty years ago, but only in recent years have the details of the molecular basis of this disease begun to be discovered. In 1993, Carstea et al (1) first documented the linkage of NPC to human chromosome 18. In 1996, complementation studies indicated that at least two genes could be implicated in causing the NPC phenotype (2). The most common complementation group (major form) is now designated as NPC1 and accounts for greater than 95% of all human NPC cases. The human NPC1 gene has been mapped to chromosome 18q11, and has been identified and sequenced (3). Numerous different mutations have been documented in NPC patients and many of the mutations are clustered in the terminal third of the gene(4). The gene responsible for the NPC phenotype in two murine models is linked to the mouse chromosome 18, which is syntenic with areas of human chromosomes 18 and 5 (5).

A feline model of NPC has been characterized and is phenotypically, morphologically, and biochemically identical to human NPC1 (6, 7). Complementation studies using cultured fibroblasts from NPC affected cats and NPC1 affected humans suggest that the gene responsible for the NPC phenotype in this colony of cats is orthologous to the gene responsible for the major form of human NPC, NPC1 (8). Characterization of the feline NPC cDNA was performed to confirm that the gene was an ortholog of human *NPC1* and to identify the mutation responsible for the NPC phenotype in this feline model. The hypotheses for this work were that feline Niemann-Pick Disease Type C involves the gene that is orthologous to the NPC1 gene in humans, and a mutation in this gene results in the NPC phenotype in this cat colony.

MATERIALS AND METHODS

Feline NPC cDNA sequence and mutation analysis

RNA was extracted from frozen liver (20mg) or cultured fibroblasts (confluent T25 flask) using the RNeasy mini kit (QIAGEN, Santa Clara, CA). Extracted RNA (12.5µl) was used for cDNA synthesis (Advantage RT-for-PCR kit, CLONTECH, Palo Alto, CA) (CLONTECH kit #K1402-1, Palo Alto, CA). First strand cDNA (5µl) was used for subsequent PCR reactions (CLONTECH kit #K1905-1, Palo Alto, CA). Human NPC1–specific PCR primers were kindly provided by Drs. Eugene Carstea and Jill Morris. Initially, 36 different human NPC primer pair combinations were used with feline

fibroblast-derived cDNA template. Standard PCR protocols were used. Of these primer pairs, eight amplified bands that were of the expected size based on the known human sequence. From these initial eight primer pair combinations that amplified bands, three primer pair combinations were chosen that incorporated sections of the 5', middle, and 3' regions of the cDNA sequence. These three primer pairs amplified single, discrete bands of the expected molecular weight. The three amplified fragments were cloned into the pCR II-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Ligated products were transformed into competent Topo-10 cells, and 100µl of the cell culture was plated on LB-plates with 50µg/ml of ampicillin. Colonies with inserts (white colonies) were selected. Colonies were cultured overnight in LB medium containing 50µg/ml of ampicillin. Plasmid DNA was isolated (Qiagen Miniprep kit #27104, Santa Clara, CA). Plasmids were digested with EcoR1, run out on a 1.5% agarose gel in 1XTAE for 30 min, and then evaluated for the expected inserts. Clones were sequenced using Sanger di-deoxy chain termination methodology on an ABI 377 Automated DNA Sequencer. ABI sequence data files were analyzed using ABI Sequence Navigator software (version 1.0.1). Based on the sequence data, feline specific primers were generated (Table 4.1), and the majority of the open reading frame of the feline NPC cDNA was subsequently cloned and sequenced (Figure 4.1). Multiple runs in both directions for each cat were used to generate a consensus sequence. Redundant

sequence data were generated from three NPC affected cats, four NPC obligate heterozygote cats and three normal control cats.

Identification of Heterozygotes

Whole blood was collected in EDTA, and 500µl was used for DNA extraction (Qiagen kit #51104, Santa Clara, CA) then used for subsequent PCR and restriction digests. Feline specific PCR primers were used to amplify an approximately 103 base-pair fragment that incorporates the mutation site. The primary PCR products were then digested with the restriction enzyme Mwo1 and subsequently analyzed by gel electrophoresis. The NPC mutation results in an unrecognizable restriction site for restriction enzyme Mwo1. Thus, no cutting occurs when the NPC mutation is present.

The Mwo1 Restriction Site:

GCNNNNN NN**G**C

CGNN NNNNNCG

The bold "G" is the mutation site in feline NPC, the underlined 'G" is the base that is changed from a "T" in the feline sequence to a "G" with an engineered primer used for the PCR reaction. The "space" denotes the restriction site for Mwo1. Primers used for amplification were:

(sense) 5'-CTC-TAGG-TAC-CCG-AAT-AGG-CTT-TGC-3'

(antisense) 5'-CGA-TCG-GTG-CTG-TTG-TAG-**C**CT-CTA-3'

A thymidine nucleotide at 2879 location (9 nucleotides downstream of mutation site) changed to guanidine (underlined "C" above) to provide a MwoI site with normal sequence.

DNA extracted from whole blood was used in PCR reactions to amplify the ~105bp fragment that spans the mutation site. Primers used for the carrier assay are identified above. PCR conditions were as follows: initial denaturing for 3 min. at 95°C, then 30 cycles of (95°C for 1 minute / 56°C for 45 seconds / 72°C for 1 minute). The reaction contained 3% DMSO. PCR product (10µl) was used for the MwoI digestion. The restriction digest included 0.5µl of the restriction enzyme, 2.5µl of the provided buffer and 12µl of distilled water, for a total of 25µl. The restriction digest was run for 2 hours at 55°C. The entire 25µl sample plus 5µl of marking dye was loaded onto a 3% Metaphor gel with ethidium bromide and run in 1X TBE buffer at 90v for 1.5-3 hours. Final bands were visualized with an ultraviolet light source.

The PCR based assay for the identification of heterozygotes was used to screen all cats currently in the NPC colony. Additionally, RNA was extracted from frozen fibroblasts of deceased animals using the RNeasy mini kit (QIAGEN, Santa Clara, CA) then cDNA was synthesized (Clontech kit #K1905-1, Palo Alto, CA) and evaluated with the assay.

COLLABORATIVE STUDIES

Karyotyping

Karyotyping of NPC obligate heterozygotes and NPC affected cats was performed in collaboration with Dr. Elizabeth McNiel, Colorado State University. Metaphase slides for karyotyping were made from pokeweed stimulated peripheral blood lymphocytes. Chromosomes were stained with DAPI and Chromomycin A3 and R-banding was enhanced with a computer imaging system (9). Chromosome number was determined from 20 metaphase spreads. Full karyotypes were performed for 5 metaphase spreads. R-banded metaphase chromosome spreads of normal and known heterozygotes were generated. Comparisons of maternal to paternal chromosome pairs as well as to existing normal-cat metaphase chromosomes were made.

Cytogenetic localization of Feline NPC

Fluorescence in situ hybridization (FISH) analysis using feline PAC clones was conducted in collaboration with the laboratory of Dr. Stephen O'Brien (National Cancer Institute, Frederick, MD). Feline PAC clones were labeled with biotin-11 (Sigma) by nick translation (10) and the final probe size of less than 500bp was verified on a 1.2% gel with appropriate markers . Metaphase spreads of domestic cat (FCA#215) were prepared by standard cytogenetic techniques (11) and FISH analysis was performed. (12). Briefly, the metaphase spreads were denatured in 70% formamide 2XSSC in an

80C oven for 90 seconds and dehydrated in cold 70%, 90% and 100% ethanol. 400ng of labeled probe, 10ug of salmon sperm carrier DNA, and 3ug of cat COT-1 DNA were resuspended in 50% formamide-10% dextran sulfate-2XSSC and denatured for 10 minutes at 75C. The denatured probe cocktail was layered on the denatured metaphase chromosomes and placed in a humidifying box at 37C. Following 48 hours of incubation, and post-hybridization washes, the hybridized biotin labeled probes were detected with fluorescein isothioscyinate (FITC) conjugated to avidin DCS (5mg/ml, Vector Labs).

Fluorescence signals were captured as gray scale images using Zeiss Axioskop epi-fluorscence microscope with a cooled charged couple device camera (Photometrics CE 250), and Apple Macintosh power PC, and the Oncor Image system. Gray scale images were computer enhanced, pseudcolored, and merged using Oncor image software. Images of Dapi stained chromosomes were merged with FITC detected signals allowing for direct visualization, chromosome identification and chromosome assignment.

NPC Immunoreactivity Evaluation

Studies using rabbit anti-mouse NPC1 antibody and anti-human NPC1 antibody were performed in collaboration with W. Sherman Garver, (University of Arizona College of Medicine, Tucson, Arizona. Immunoblot analyses using immune and preimmune serum on normal feline liver homogenates were conducted.

Immunoblot analysis

Frozen liver from a normal cat was homogenized on ice in 0.32 molar sucrose. Homogenates were electrophoresed through SDS/5% polyacrylamide gels, transferred to nitrocellulose membranes, and Western blotted for NPC1 using rabbit anti-human and anti-mouse NPC1 polyclonal antibodies. Immunoreactive bands were visualized using a labeled goat anti-rabbit secondary IgG and enhanced chemiluminescence detection.

RESULTS

cDNA and mutation analysis

To date, 97% of the estimated 3834 base pair cDNA sequence has been determined. The feline nucleotide sequence has greater than 86% homology with the human sequence while the predicted feline amino acid sequence has greater than 90% identity with the human amino acid sequence (Figure 4.2). A single base substitution of guanine for cytosine (G2870C) was identified in all NPC affected cats evaluated (n=5). Carriers were heterozygous for the same allele (Figures 4.3 - 4.5). This point mutation results in an amino acid substitution of a conserved cysteine for serine (C957S) (Figure 4.6).

Heterozygote Identification

The Mwo1 restriction site is present in normal cats and both copies of the NPC gene is cleaved, resulting in a single visual band at approximately 80bp. NPC affected cats are

homozygous for the mutation at the restriction site, resulting in no cleavage of either copy, thus a single band is visualized at approximately 103bp. Feline NPC heterozygotes can be identified by the presence of two bands, one that has been cut (wild type~80bp) and one that remains uncut (mutant~103bp) (Figure 4.7). All previously known obligate heterozygotes and NPC affected cats were correctly identified (NPC affected N=3, NPC obligate heterozygote N=4, Normal N=4). An additional 33 cats from the NPC colony were screened; two additional female and five additional male carriers were identified.

COLLABORATIVE STUDIES

Karyotyping

Mutations resolvable at the cytogenetic level were not detected in NPC obligate heterozygotes or NPC affected cats (Figure 4.8). The normal karyotypes supported a small mutation.

Chromosomal Localization

Hybridization data using feline NPC PAC localized the NPC-1 gene to chromosome D3q1 (Figure 4.9).

The feline NPC1 protein and antibody investigations

Immunoblot analysis of liver homogenates from normal cats revealed that no cross-reaction with the feline NPC protein was evident with either the rabbit anti-human NPC1 or rabbit anti-murine NPC1 antibodies.

DISCUSSION

Characterization of the feline NPC1 cDNA sequence confirmed that the gene responsible for the NPC phenotype in the feline model is indeed the ortholog of the human NPC1 gene. Strong nucleotide and amino acid sequence homology exists between the cat and human, which is actually greater than that between the human and mouse. This is not surprising given that comparative genomic studies have shown that the cat is phylogenetically more closely related to humans than the mouse, and that the overall feline-human syntenic chromosomal conservation is greater than the mouse-human (13). This degree of homology is underscored by the high efficiency of the human based primers used in the initial PCR amplification of the feline cDNA sequence. The feline NPC gene localized to chromosome D3; D3 has been shown to be homologous to human chromosome 18, the chromosome to which the human NPC1 gene has been localized (1, 13).

The position of the single missense mutation (G2870C) identified in this colony is consistent with reports of several mutations in people involving the terminal third of the

NPC1 gene(14). Over thirty different mutations of the NPC1 gene have been identified in humans(3, 15, 16). Given the fact that this is an inbred colony of cats from a single queen and son mating, arising from a single ancestor, genetic heterogeneity was not expected. This is similar to the founder effect identified in the allelic variant of NPC, Niemann-Pick disease type D (NPD), identified in Nova Scotia (17).

The NPC1 gene codes for a 1278 amino acid protein. The putative NPC1 protein is predicted to have thirteen transmembrane regions and three large hydrophilic loops (18) (Figure 4.10). The NPC1 protein has been shown to reside in late endosomes and to transiently interact with lysosomes and the trans-Golgi network (19). The exact function of the NPC1 protein remains unknown. However, it has been shown to be a transport protein that is critical for the movement of endocytosed material (19, 20). Several thoughts on the possible functions of the NPC1 protein have been proposed and include 1) direct transporting that facilitates the movement of cholesterol out of the lysosomes, 2) a docking/fusion role that mediates the interaction of cholesterol-laden vesicles and endosomes, or 3) acting as a molecular pump (18). The mechanism(s) by which the mutation in the NPC1 gene and resultant C957S amino acid substitution leads to disease pathogenesis in NPC remains unknown. The 957 cysteine residue is conserved across multiple species and is likely critical to the structure and subsequent function of the NPC1 protein (21).

The pathogenesis of the brain dysfunction in NPC is still not understood. It is clear that outside the central nervous system (CNS), the NPC1 lesion results from a processing defect of LDL-derived cholesterol, but it is widely accepted that exogenous cholesterol is not presented to the CNS because of the blood-brain barrier. Thus, the NPC1 protein must have other functions, at least within the CNS, which have yet to be identified. Overall, the NPC1 protein is not closely related to any other known proteins, but the membrane orientation of the NPC1 sterol sensing domain (SSD) is identical to those of HMG CoA reductase and SCAP, known mediators of cholesterol homeostasis (22).

The development of a PCR assay to identify feline NPC heterozygotes has greatly facilitated colony management and should result in an increased number of affected kittens produced. The ability to rapidly and accurately genotype offspring is a significant improvement over previous methods, composed of breeding trials and filipin staining of cultured fibroblasts to determine heterozygosity. The maintenance of this colony and the availability of affected animals will be valuable for further characterization of the NPC molecular defect and evaluation of potential treatment modalities.

Sequence analysis of the 5' end of the feline NPC1 cDNA has not been completed. Efforts are currently underway to isolate the remaining 5' end of the feline cDNA sequence (approximately 100bp). Thus far, 3734 bases of the anticipated 3834 base

pair cDNA have been identified. Early attempts have relied on primers based on the known human and mouse NPC1 cDNA sequence, but the 5' region tends to have less homology across species and the area is often G:C rich, making amplification of this area more difficult. Future efforts to isolate the 5' end of the cDNA will utilize 5' SMART RACE (CLONTECH, Palo Alto, CA) and/or the feline PAC clones generated in Stephen O'Brien's laboratory.

Studies conducted with rabbit anti-human NPC1 and anti-mouse NPC1 antibodies resulted in no reaction with the feline NPC1 protein. This was not surprising given that the antibodies were generated against 19 amino acid residues in the human and mouse, each of which showed only partial sequence homology to the predicted feline amino acid sequence (15 of 19 in the human and14of 19 in the mouse). A suitable region of the predicted amino acid sequence of the feline NPC protein against which to generate antibodies was identified. The residues are in the same region as those used for the development of the anti-mouse NPC1 and anti-human NPC1 antibodies, which have both proven reactive (20, 23). A feline NPC specific polyclonal antibody has been ordered from Research Genetics (Huntsville, Alabama) and will be available by the end of 2000.

Although the exact function of the NPC1 protein remains unknown, the field is rapidly advancing and it is probable that the elucidation of the function(s) of the NPC1 protein will be determined in the very near future. These discoveries will play a pivotal role in understanding disease pathogenesis in NPC, in both the peripheral tissues and the CNS, and are vital to the development of rational treatment modalities.

REFERENCES

1. Carstea, E. D., Polymeropoulos, M. H., Parker, C. C., Detera-Wadleigh, S. D., O'Neill, R. R., Patterson, M. C., Goldin, E., Xiao, H., Straub, R. E., Vanier, M. T., and et al. (1993) Linkage of Niemann-Pick disease type C to human chromosome 18. *Proc Natl Acad Sci U S A* 90, 2002-2004

2. Vanier, M. T., Duthel, S., Rodriguez-Lafrasse, C., Pentchev, P., and Carstea, E. D. (1996) Genetic heterogeneity in Niemann-Pick C disease: a study using somatic cell hybridization and linkage analysis. *Am J Hum Genet* 58, 118-125

3. Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B., Nagle, J., Polymeropoulos, M. H., Sturley, S. L., Ioannou, Y. A., Higgins, M. E., Comly, M., Cooney, A., Brown, A., Kaneski, C. R., Blanchette-Mackie, E. J., Dwyer, N. K., Neufeld, E. B., Chang, T. Y., Liscum, L., Tagle, D. A., and et al. (1997) Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis [see comments]. *Science* 277, 228-231

4. Greer, W., Dobson, MJ, Girouard, GS, et al (1999) Mutations in NPC! Highlight a Conserved NPC1-Specific Cystiene-Rich Domain. *American Journal of Human Genetics* 65, 1252-1260

5. Loftus, S. K., Morris, J. A., Carstea, E. D., Gu, J. Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M. A., Tagle, D. A., Pentchev, P. G., and Pavan, W. J. (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene [see comments]. *Science* 277, 232-235

6. Brown, D., Thrall, MA, Walkley, SU, Wenger, DA, Mitchell, TW, Allison, RW, Smith, MO, Royals, KL, March, PA. (1994) Animal Model of Human Disease: Feline Niemann-Pick Disease Type C. *Comparative Pathology Bulletin* XXVI

7. Brown, D. E., Thrall, M. A., Walkley, S. U., Wenger, D. A., Mitchell, T. W., Smith, M. O., Royals, K. L., March, P. A., and Allison, R. W. (1994) Feline Niemann-Pick disease type C. *Am J Pathol* 144, 1412-1415

8. Somers, K. L., Wenger, D. A., Royals, M. A., Carstea, E. D., Connally, H. E., Kelly, T., Kimball, R., and Thrall, M. A. (1999) Complementation studies in human and feline Niemann-Pick type C disease. *Mol Genet Metab* 66, 117-121

9. Christian, A., McNiel, E., Robinson, J., Drabek, R., LaRue, S., Waldren, C., and Bedford, J. (1998) A versatile image analysis approach for simultaneous

chromosome identification and localization of FISH probes. *Cytogenet Cell Genet* 82, 172-179

10. Brigati, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C. K., Hsiung, G. D., and Ward, D. C. (1983) Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. *Virology* 126, 32-50

11. Modi, W. S., Nash, W. G., Ferrari, A. C., and O'Brien, S. J. (1987) Cytogenetic methodologies for gene mapping and comparative analyses in mammalian cell culture systems. *Gene Anal Tech* 4, 75-85

12. Lichter, P., Tang, C. J., Call, K., Hermanson, G., Evans, G. A., Housman, D., and Ward, D. C. (1990) High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. *Science* 247, 64-69

13. O'Brien, S. J., Wienberg, J., and Lyons, L. A. (1997) Comparative genomics: lessons from cats. *Trends Genet* 13, 393-399

14. Greer, W. L., Dobson, M. J., Girouard, G. S., Byers, D. M., Riddell, D. C., and Neumann, P. E. (1999) Mutations in NPC1 highlight a conserved NPC1-specific cysteine-rich domain. *Am J Hum Genet* 65, 1252-1260

15. Greer, W. L., Riddell, D. C., Gillan, T. L., Girouard, G. S., Sparrow, S. M., Byers, D. M., Dobson, M. J., and Neumann, P. E. (1998) The Nova Scotia (type D) form of Niemann-Pick disease is caused by a G3097-->T transversion in NPC1. *Am J Hum Genet* 63, 52-54

16. Yamamoto, T., Nanba, E., Ninomiya, H., Higaki, K., Taniguchi, M., Zhang, H., Akaboshi, S., Watanabe, Y., Takeshima, T., Inui, K., Okada, S., Tanaka, A., Sakuragawa, N., Millat, G., Vanier, M. T., Morris, J. A., Pentchev, P. G., and Ohno, K. (1999) NPC1 gene mutations in Japanese patients with Niemann-Pick disease type C. *Hum Genet* 105, 10-16

17. Greer, W. L., Riddell, D. C., Byers, D. M., Welch, J. P., Girouard, G. S., Sparrow, S. M., Gillan, T. L., and Neumann, P. E. (1997) Linkage of Niemann-Pick disease type D to the same region of human chromosome 18 as Niemann-Pick disease type C. *Am J Hum Genet* 61, 139-142

18. Ioannou, Y. A. (2000) The Structure and Function of the Niemann-Pick C1 Protein. *Mol Genet Metab* 71, 175-181

19. Higgins, M. E., Davies, J. P., Chen, F. W., and Ioannou, Y. A. (1999) Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol Genet Metab* 68, 1-13

20. Neufeld, E. B., Wastney, M., Patel, S., Suresh, S., Cooney, A. M., Dwyer, N. K., Roff, C. F., Ohno, K., Morris, J. A., Carstea, E. D., Incardona, J. P., Strauss, J. F., 3rd, Vanier, M. T., Patterson, M. C., Brady, R. O., Pentchev, P. G., and Blanchette-Mackie, E. J. (1999) The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J Biol Chem* 274, 9627-9635

21. Watari, H., Blanchette-Mackie, E. J., Dwyer, N. K., Watari, M., Burd, C. G., Patel, S., Pentchev, P. G., and Strauss, J. F., 3rd (2000) Determinants of NPC1 expression and action: key promoter regions, posttranscriptional control, and the importance of a "cysteine-rich" loop. *Exp Cell Res* 259, 247-256

22. Davies, J. P., and Ioannou, Y. A. (2000) Topological analysis of Niemann-Pick C1 protein reveals that the membrane orientation of the putative sterol-sensing domain is identical to those of 3-hydroxy-3-methylglutaryl-CoA reductase and sterol regulatory element binding protein cleavage-activating protein. *J Biol Chem* 275, 24367-24374

23. Watari, H., Blanchette-Mackie, E. J., Dwyer, N. K., Glick, J. M., Patel, S., Neufeld, E. B., Brady, R. O., Pentchev, P. G., and Strauss, J. F., 3rd (1999) Niemann-Pick C1 protein: obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. *Proc Natl Acad Sci U S A* 96, 805-810

Table 4.1

Overlapping Primer Pairs Used for Sequencing of the Feline NPC Gene

#	Primer Pairs	Forward	Reverse	Band	Size
1	f103sn / f957asn	CATCTGGAGATAAGAGGTAC	TGCCATGTAGGTGATCCAC	A	854
2	3F / f1197asn	CTGCCTCTACAGTTTCTGTCCA	GGGAGAAGAAGATGATGGG	B	809
3	RTEsn / RTGasn	GCTTGGACGCCATGTAT	AGCACGGAATGGCTGTT	C	693
4	f1366sn / f2435asn	ACACACTTACCAGCCGTACC	TGAAGAAGGGAGTCGATGAG	D	1070
5	f2447sn / 18R	TACCAGCGAGATGAACGTC	GCGTTAAATATCTGCTGCACCT	E	653
6	f2787sn / f3531asn	TGTGTACTTTGTCCTGGAGG	TGACCAAGATCATAGCGATG	F	744
7	f3370sn / f3997asn	TGTCTTCTACGAACAGTACCTG	ACGACCCACAGACACAGTTC	G	627
8	F1755sn / f2329asn	CTTGTGTTAGGAGGCTATGATG	CATACTTAGGAGCCACTTCTCC	H	574

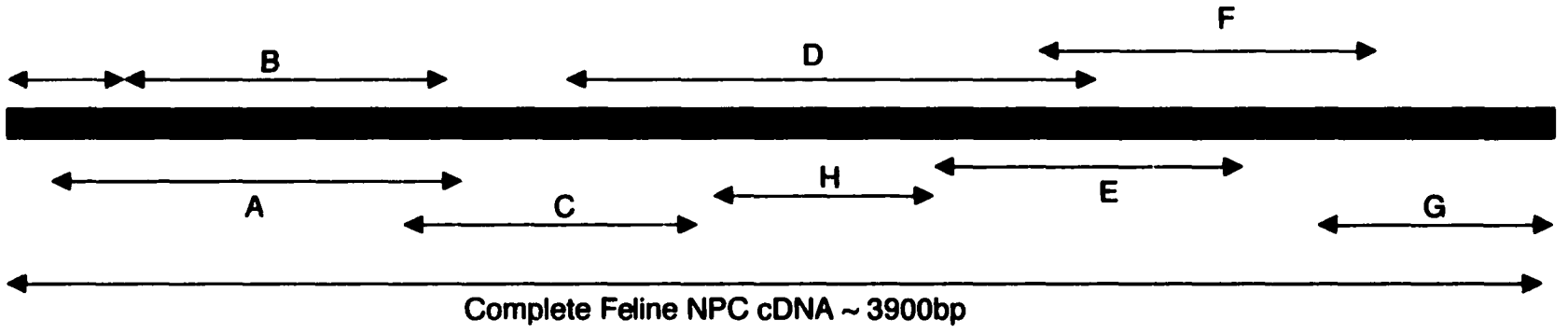


Figure 4.1

The Feline NPC cDNA

The complete feline NPC cDNA is expected to be approximately 3900bp.
Fragments for the overlapping segments are designated by letters A-H.
Details about bands A-H (size and primers used) are listed in Table 4.1
The red arrow denotes the first 103 missing bases of the expected open reading frame that remain to be identified.

Figure 4.2

Sequence data for the NPC1 gene

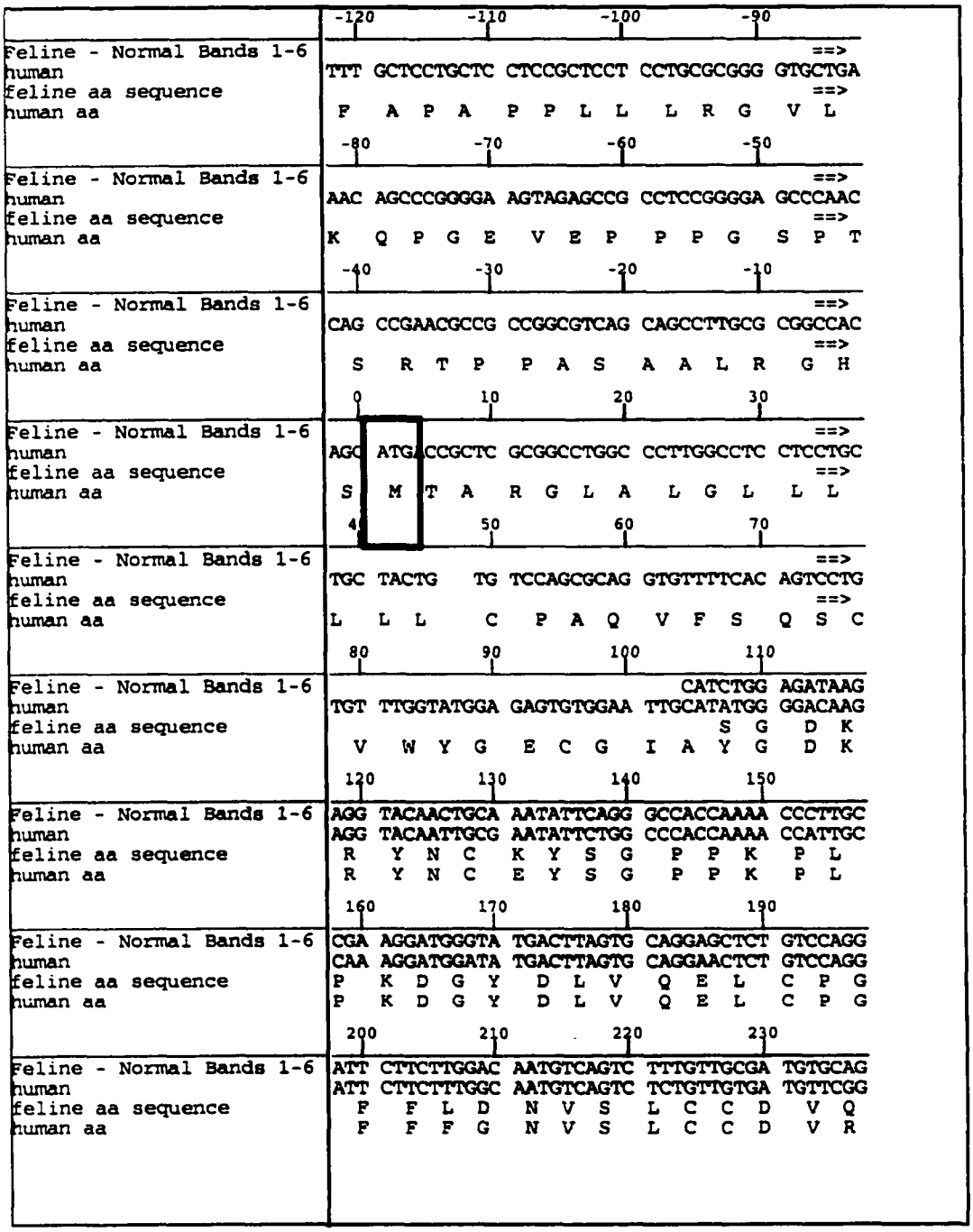
Nucleotide sequence from: normal cat (Feline-Normal Bands 1-6) and normal human (human).

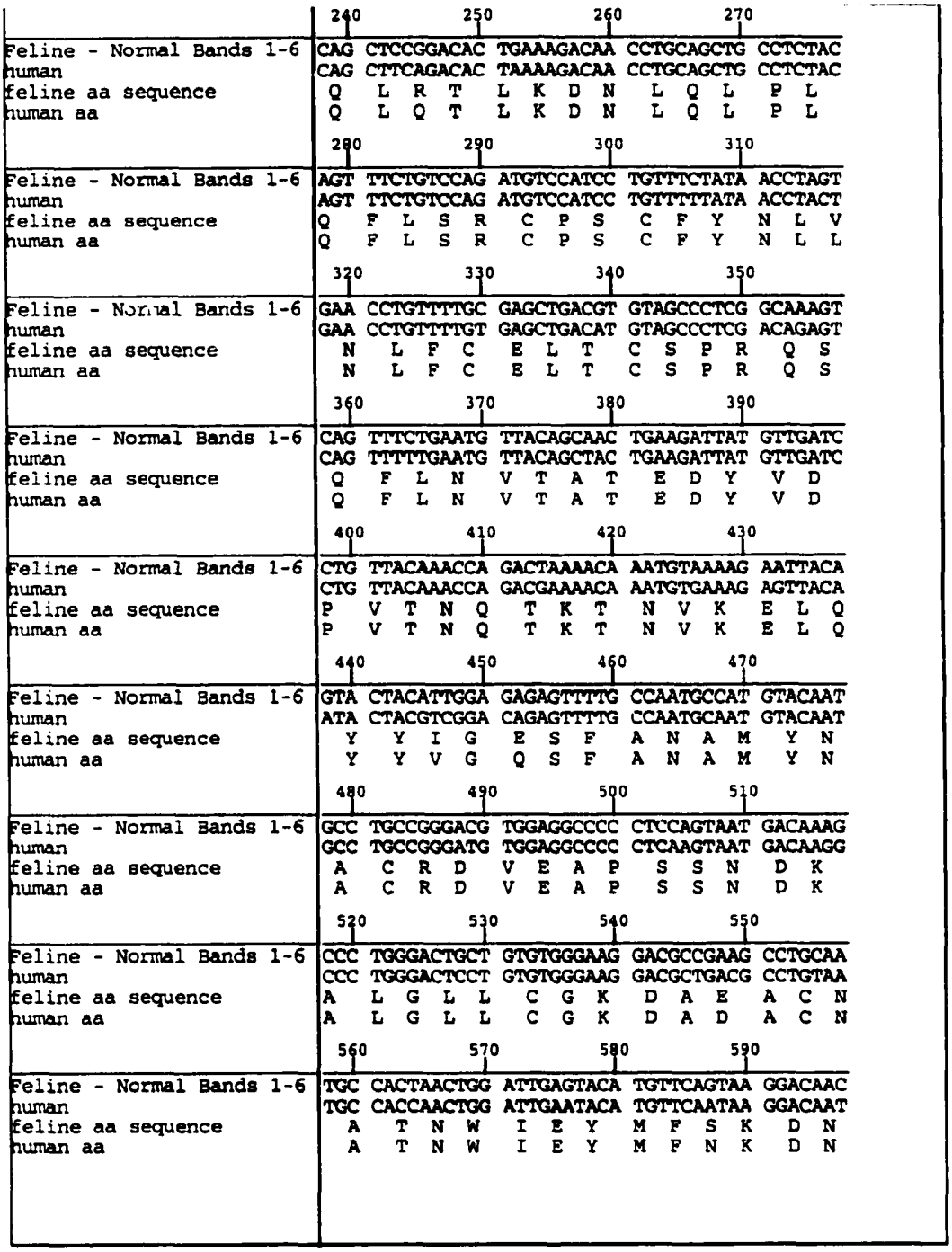
Amino acid sequence from: normal cat (feline aa sequence) and normal human (human aa)

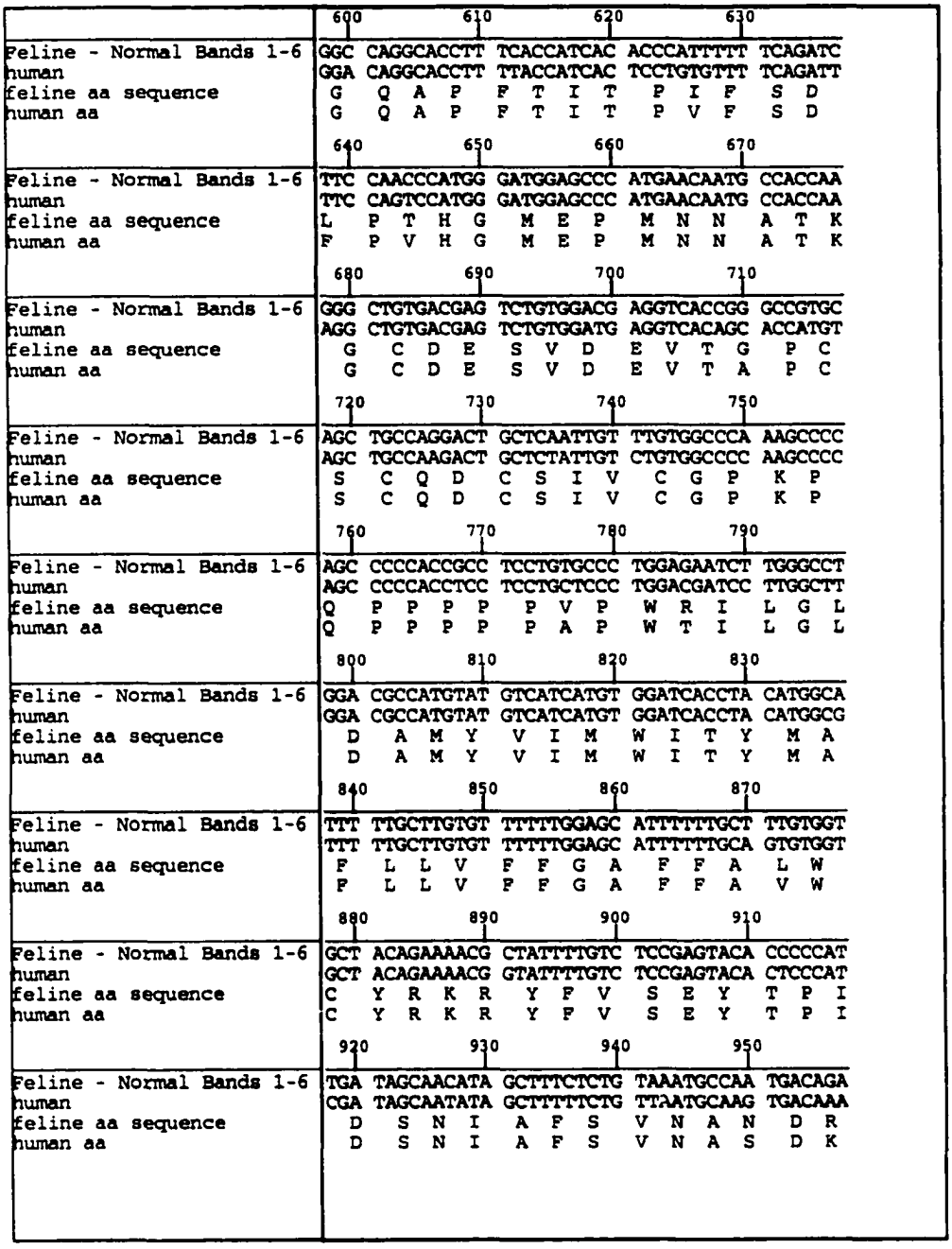
The start codon in the human sequence is designated by a blue box.

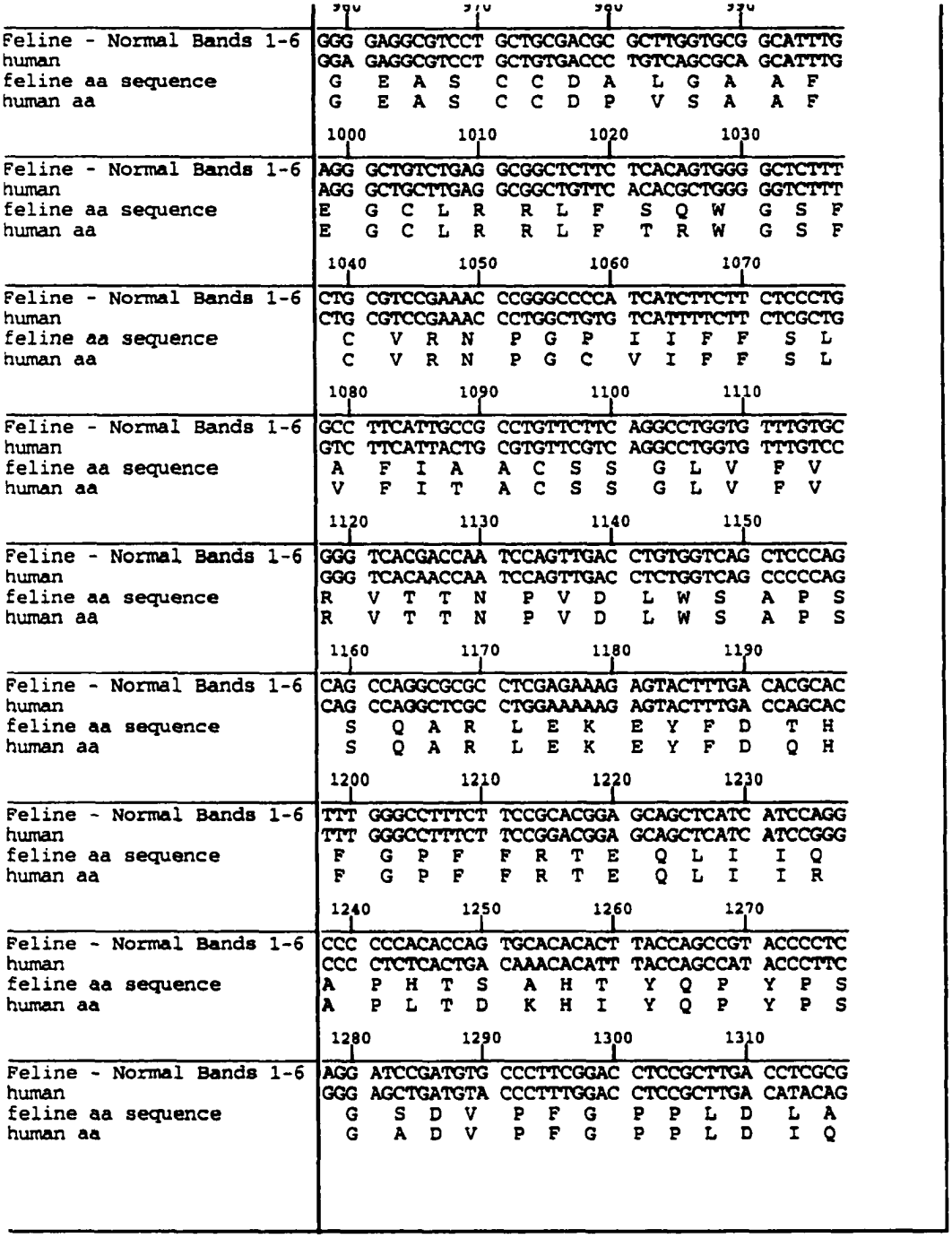
The site of the feline NPC mutation is in the orange box (nt 2870).

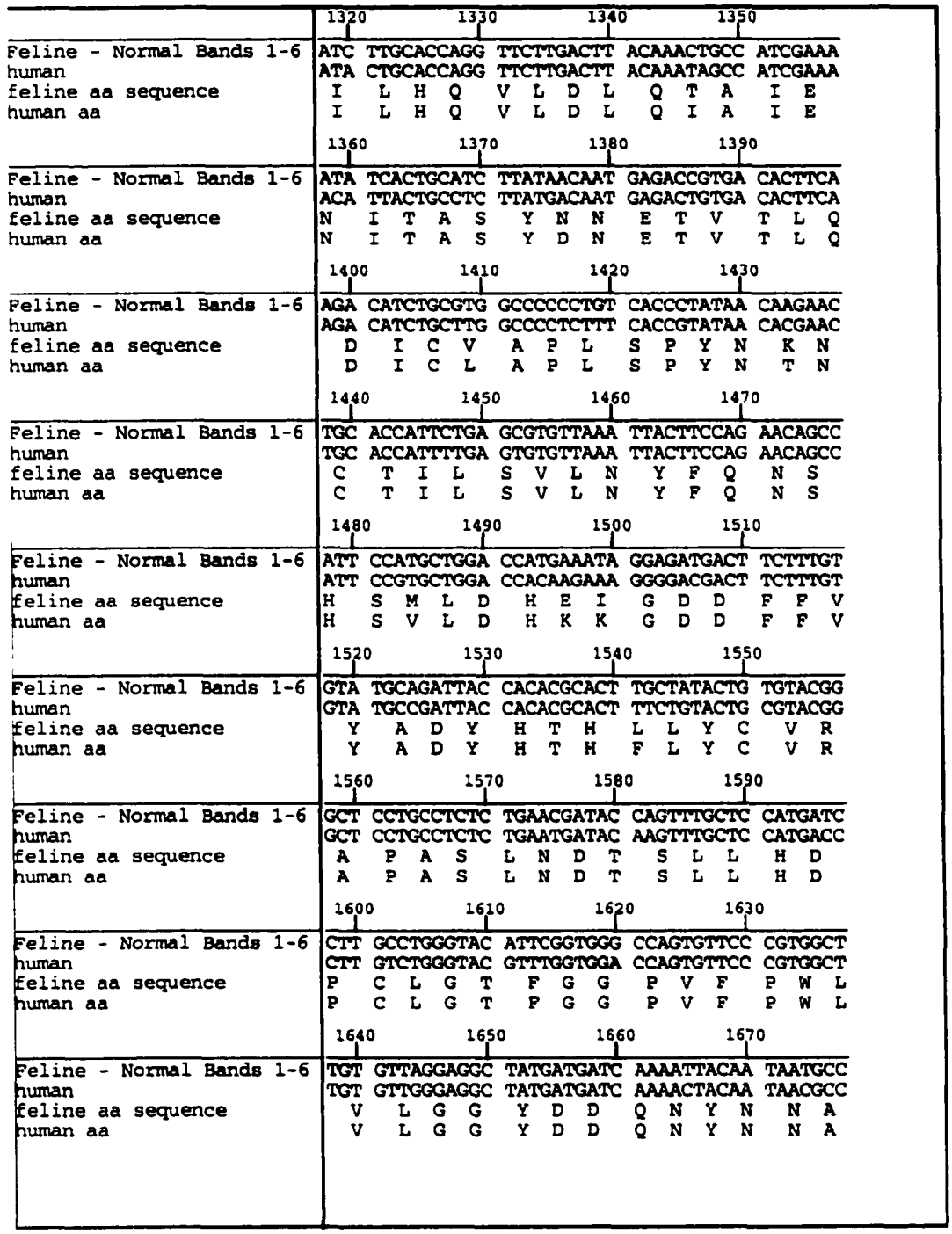
The region used to generate the feline specific anti-feline NPC1 antibody is represented by the green box (aa1257-1275).

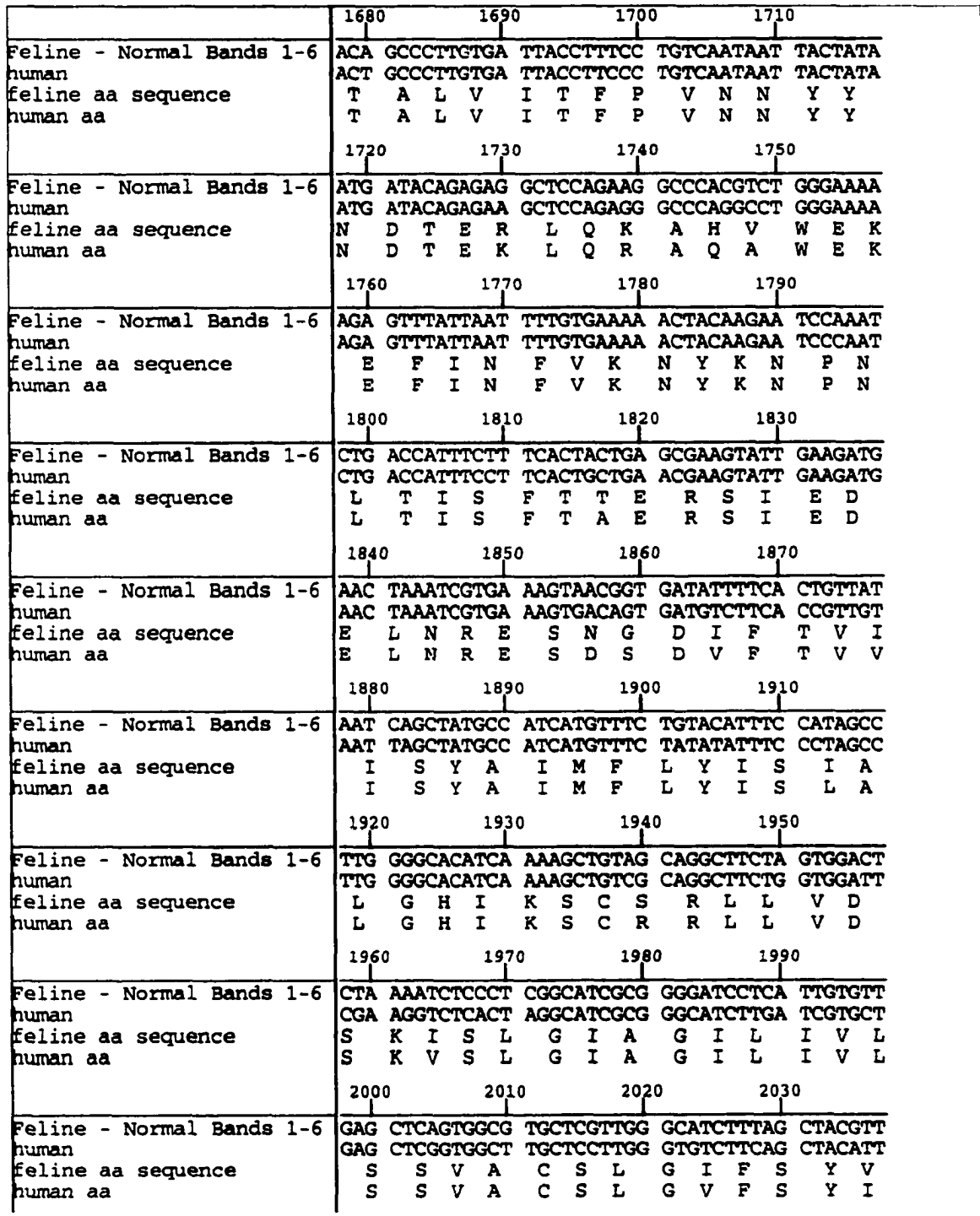


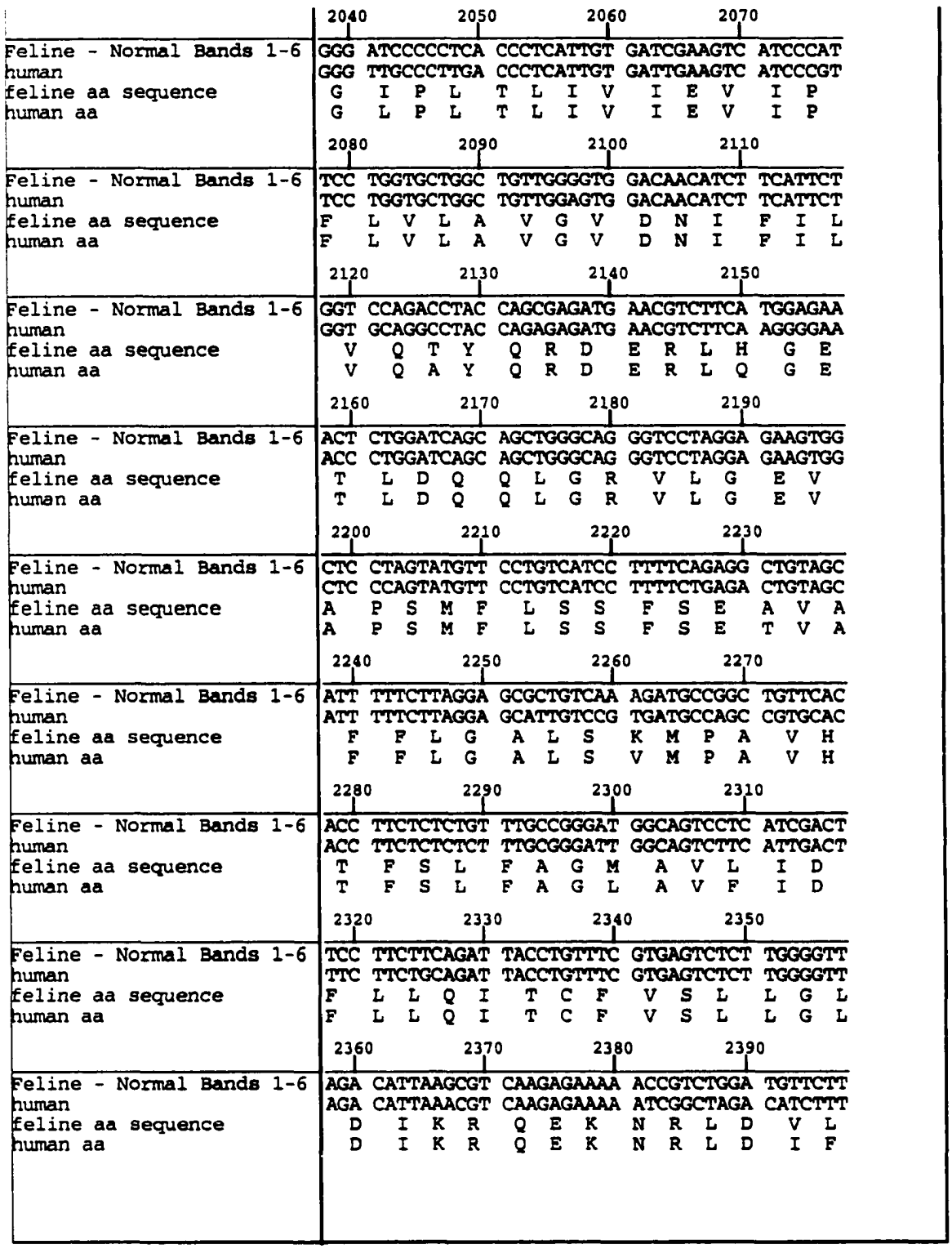


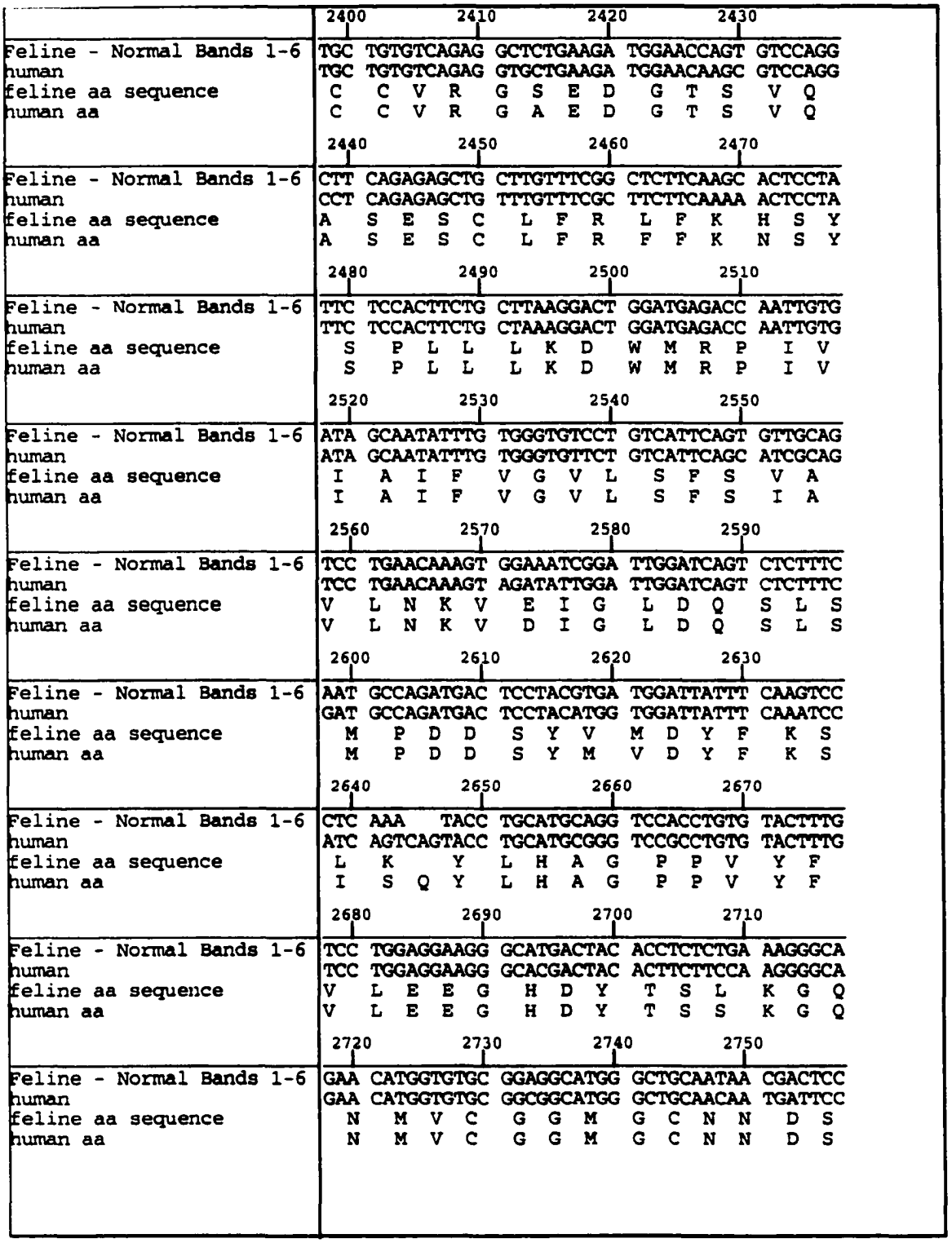


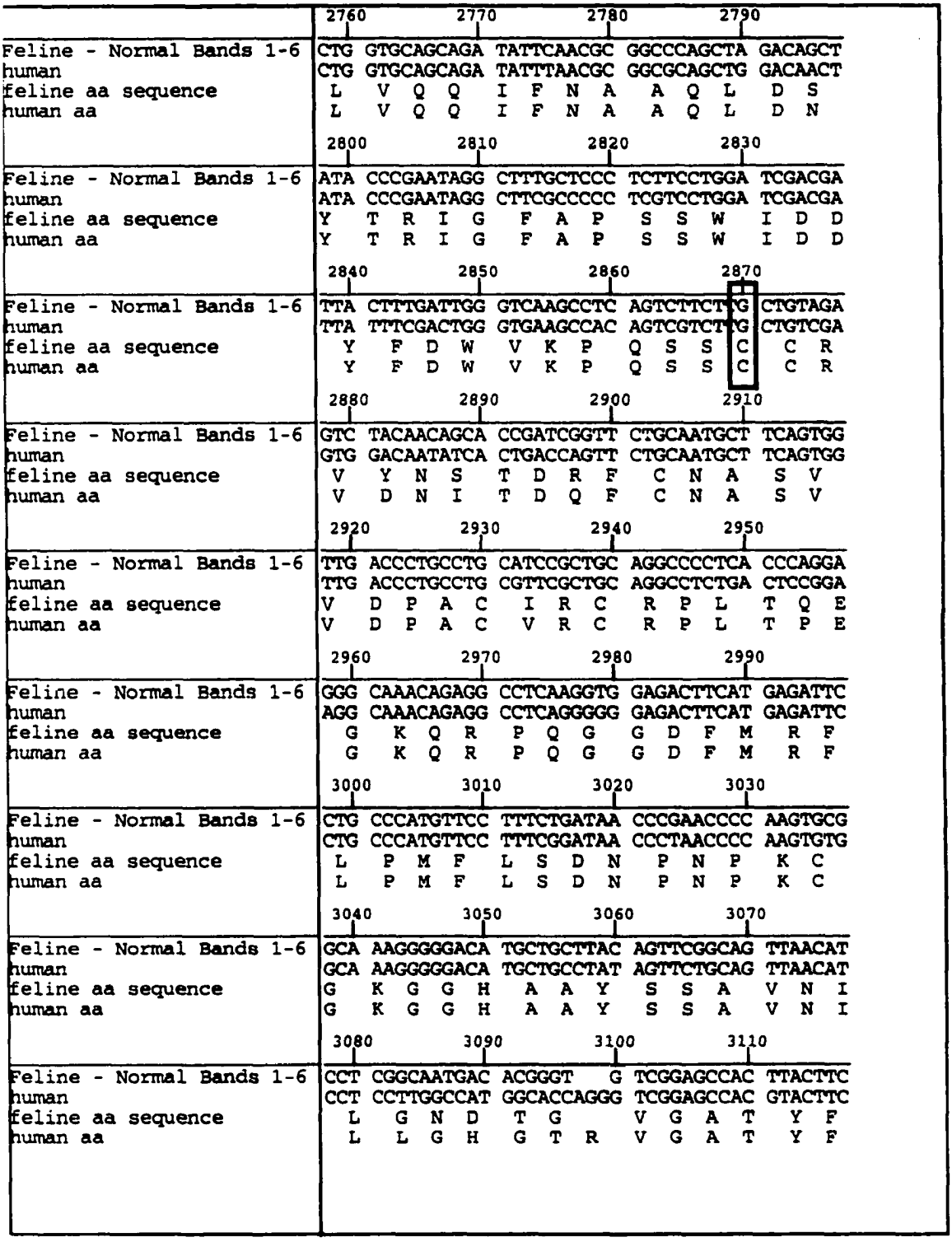


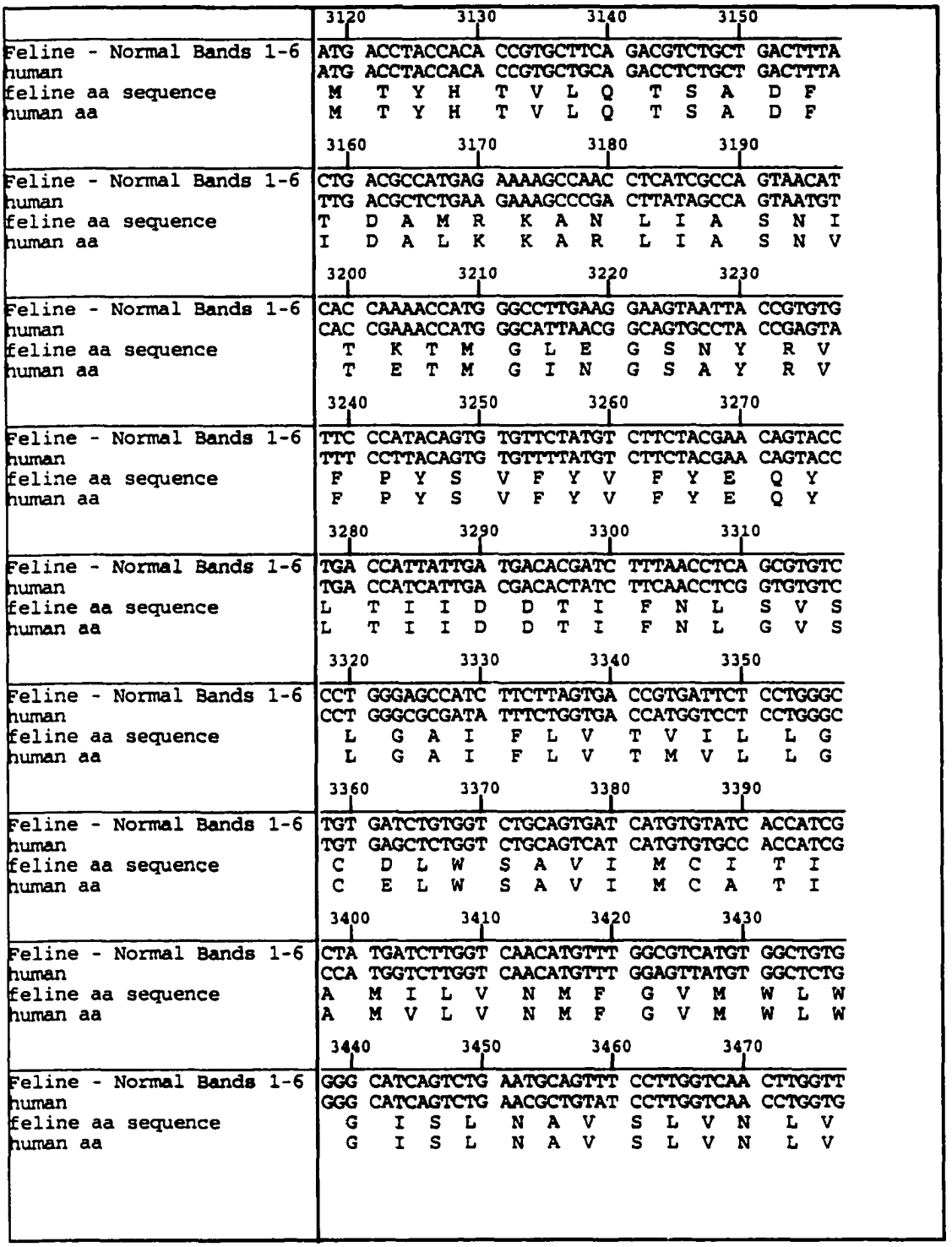












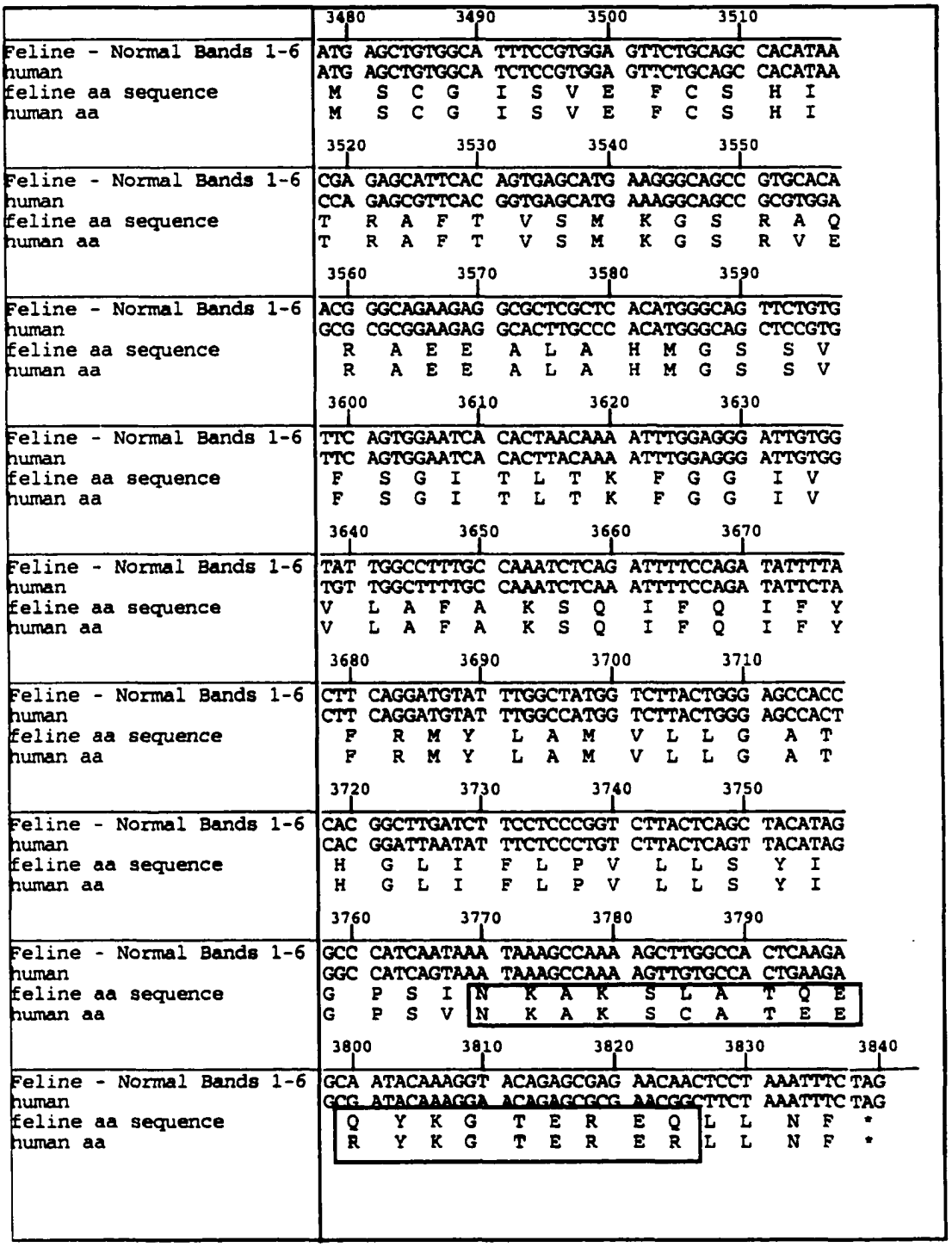


Figure 4.3

Electropherogram from normal feline sequence that is homozygous for guanine (G) at the mutation site.

4.4

Electropherogram from an NPC affect cat that is homozygous for cytosine (C) at the mutation site.

4.5

Electropherogram from an obligate NPC heterozygote that has both guanine (G) and cytosine (C) and is heterozygous at the mutation site.

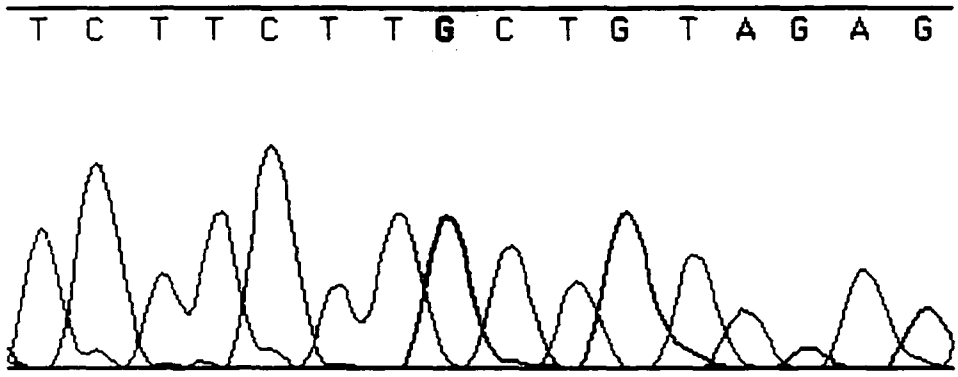


Figure 4.3 2870

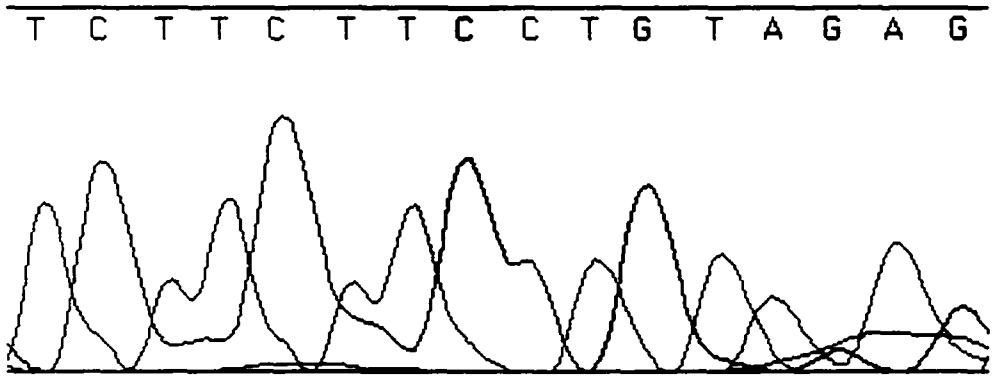


Figure 4.4 2870

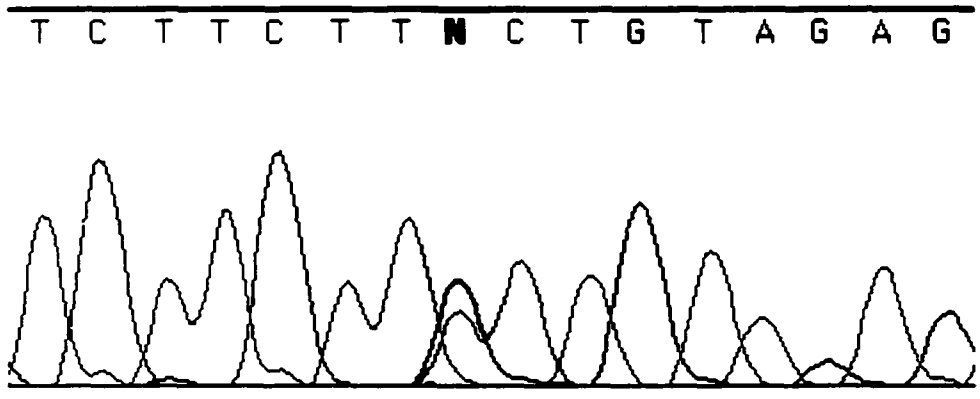


Figure 4.5 2870

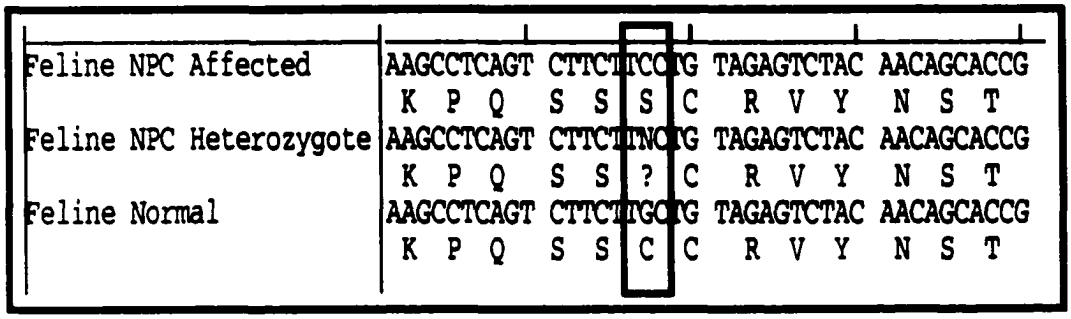


Figure 4.6

Base and amino acid sequences from NPC affected, NPC heterozygote, and normal cats.

The area in the red box represents the site of the NPC mutation and illustrates the resulting amino acid from the C957S substitution.

NPC affected cats with serine and normal cats with cysteine.

The "N" and the "?" for the NPC heterozygotes reflects the heterozygous alleles of carriers.

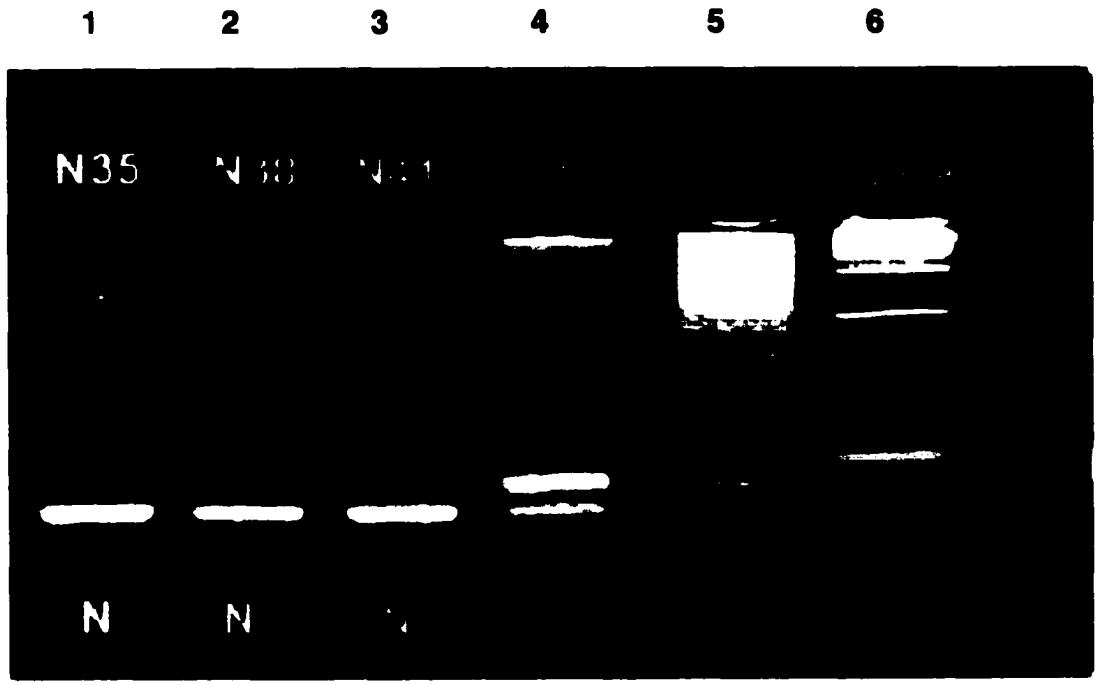


Figure 4.7

Gel from PCR based carrier assay

Lanes 1-3 represent normal genotypes with a single band at ~80bp.

Lane 4 represents a carrier with two bands, the cut wild type at~80bp and the uncut mutant at ~100bp.

Lane 5 represents a homozygous affected cat with both bands uncut.

Lane 6 is the marker.

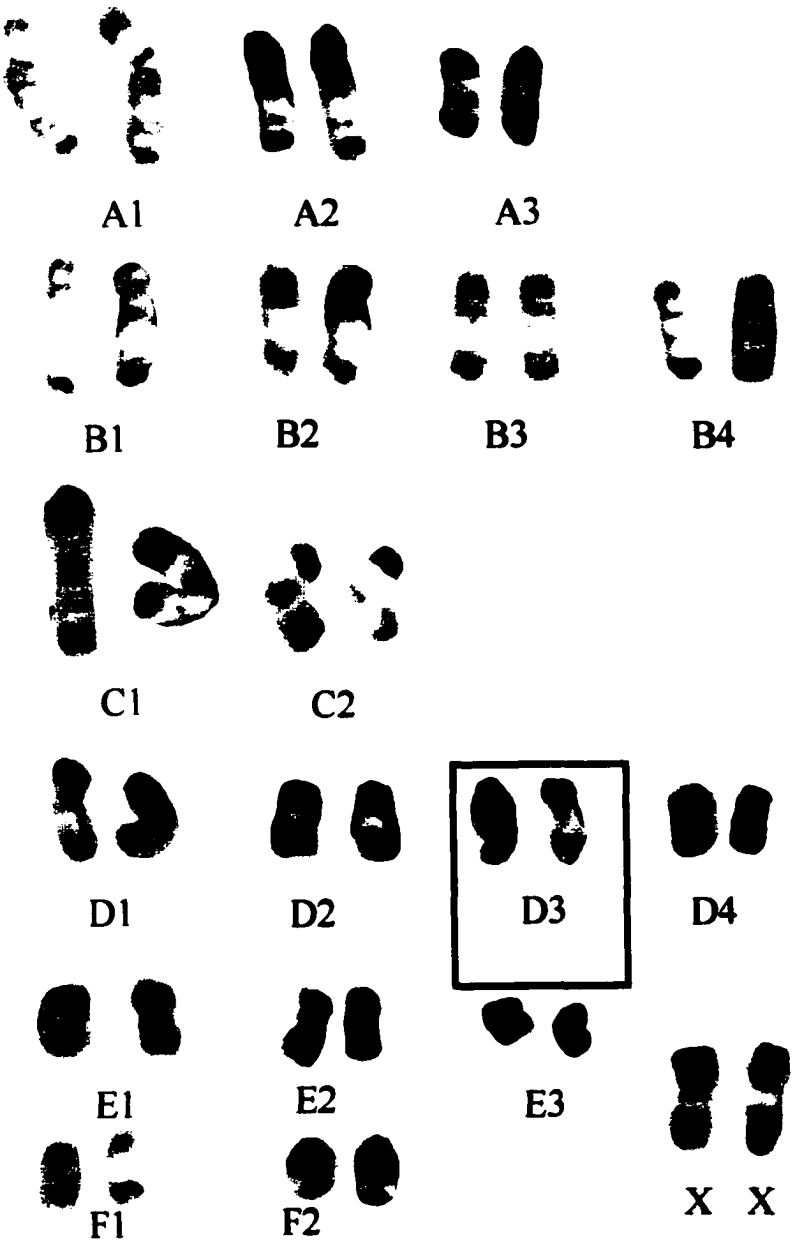


Figure 4.8

Karyotype of NPC obligate heterozygote.

D3, in the red box, is homlogous to human chromsome 18.

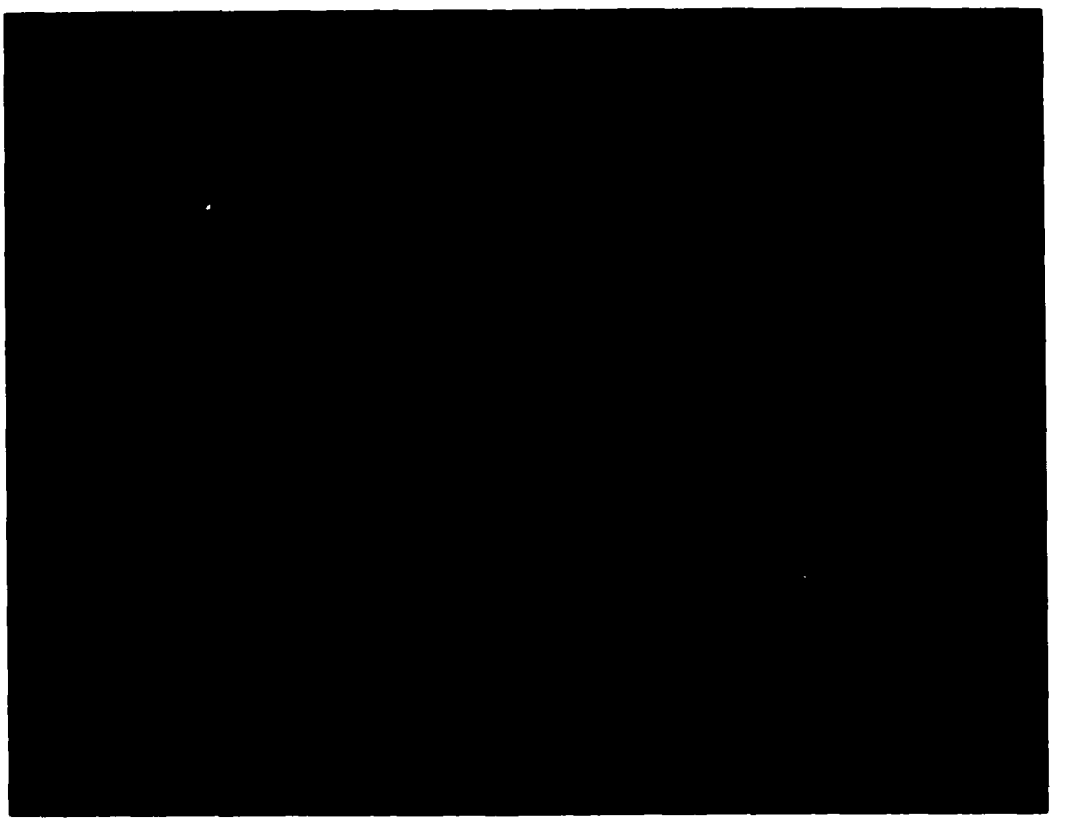


Figure 4.9

FISH analysis of the feline *NPC1* chromosomal localization.

Chromosomes outlined by yellow boxes represent D3, where the feline NPC1 gene localizes and a homolog of human chromosome 18, the localization site of the human *NPC1*

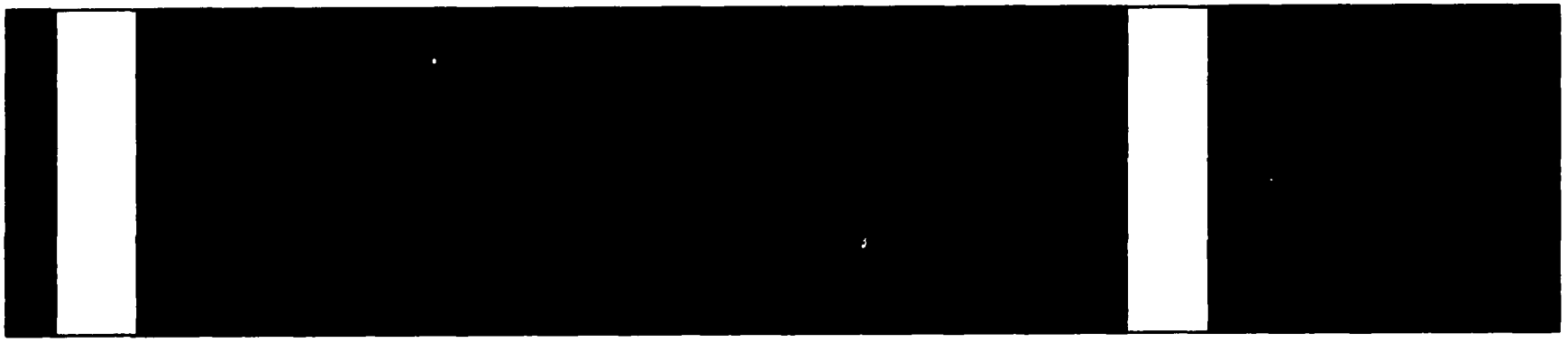


Figure 4.10

The Predicted Human NPC1 Protein

13 Transmembrane domains

NPC1 critical regions

NH_2-terminus has an endoplasmic reticulum targeting sequence

COOH-terminus a lysosomal targeting sequence