GERMAN SHEPHERD DOG DEGENERATIVE MYELOPATHY: GENETIC ANALYSIS IN A SPONTANEOUS CANINE MODEL OF DEMYELINATING DISEASE

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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To my children, Michael J. Cheeseman, Bradley D. Cheeseman and Alyssa N. Cheeseman, I dedicate this work; they have been my true inspiration

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
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To evaluate the significance of genetic variation in degenerative myelopathy (DM) of the German shepherd dog (GSD), we performed DNA analysis of the Dog Leukocyte Antigen (DLA) major histocompatibility complex (MHC) II region. We specifically looked at DRB1, DQB, DQA. Further analyses included Myelin Basic Protein (MBP), superoxide dismutase (SOD1), apolipoprotein E (APOE), interleukin-4 receptor (IL4R), hemochromatosis gene (HFE) and transforming growth factor beta-1 (TGFB1).

GSDM appears to be an autoimmune related, progressive spinal cord disease that occurs later in life. The clinical signs and pathology appear to be similar to primary progressive multiple sclerosis (PPMS) in humans. Based on the hypothesis that GSDM is an autoimmune disease closely related to PPMS in the human being, the purpose of this thesis was to further our understanding of the genetic relationship between these diseases by investigating the genetic similarities. DLA-DRB1 was found to have an allelic association in 7 of 8 GSDM dogs tested. Variations in PCR product size and banding patterns of IL4R and APOE were noted based on analysis of genomic DNA amplified with sequence specific primers. IL4R and APOE have been found to have a novel association in patients with PPMS. There were no changes noted in HFE and TGFB1, which has previously been found to be associated with relapsing remitting multiple

sclerosis (RRMS). Analysis of MBP amplified from genomic DNA showed a visible variation in the PCR product based on product size. The variation was found in 88% of DM dogs tested and only in 57% of healthy GSD. This variation is suggestive of a 70 bp tandem repeat (TGGA) deletion. This deletion correlates to the same deletion found in a population of multiple sclerosis (MS) patients in Finland.

The genetic evaluations demonstrated in this study support the fact that GSDM has a genetic predisposition. This study also indicates a disease analogous to PPMS. Therefore, this study supports GSDM as a spontaneous canine model of PPMS.

CHAPTER 1
INTRODUCTION

Clinical Significance of GSDM and PPMS

First described by Averill in 1973 Degenerative myelopathy of German shepherd (GSDM) was described as a progressive degenerative neurological disorder (Averill 1973). Symptoms include ataxia, paralysis in the lower limbs and eventually death. Although a few cases have been reported in other large breeds of dogs, the disease appears with relative frequency in the German shepherd breed, suggesting that there is a genetic predisposition for German shepherd dogs (GSD) in developing DM. The German shepherd dog has a high incidence (2.01%) in comparison to the low incidence (0.19%) of DM in other breeds as reported by a recent epidemiologic study (Oji et al., 2006). The age of onset for GSDM has been reported between 5 and 14 years (Averill 1973; Braund and Vandevelde 1978; Romatowski 1984; Barclay and Haines 1994; Johnston,Barrie et al. 2000), which corresponds to the third to sixth decades of human life. PPMS has an onset between late thirties and early forties. In both GSDM and PPMS there is no sex preference.

Primary Progressive MS is characterized by a gradual progression of spinal cord disease that may exacerbate but has no remissions (Bashir and Whitaker 1999; Montalban 2005). PPMS has a progressive onset of walking difficulties; steadily worsening motor dysfunctions and increased disability, but with a total lack of distinct inflammatory attacks. Fewer and smaller cerebral lesions, diffuse spinal cord damage, and axonal loss are the hallmarks of this form of multiple sclerosis (MS). There is continuous progression of deficits and disabilities, which may quickly level off, or continue over many months and years (Oji 2006; Ebers 2004).). However, PPMS has a shorter course than RRMS, that the course is usually 7-8 years until incapacitation. The mental status of the patient is maintained throughout the duration of the disease.

Degenerative myelopathy is characterized by wide spread demyelination and axon loss which begins in the thoracolumbar spinal cord and later involves other areas of the spinal cord (Clemmons 1989; Barclay and Haines 1994). Primary Progressive MS is characterized by a gradual progression of spinal cord disease that may exacerbate but has no remissions (Bashir and Whitaker 1999; Montalban2005). No cranial nerve deficit was reported in GSDM (Averill 1973). Primary Progressive MS may eventually progress to involve the brain, but brain damage is much less likely than RRMS or SPMS (Montalban 2005).

The pathogenesis of GSDM is currently unknown, although it is believed to be autoimmune related with a genetic basis (Waxman, Clemmons et al. 1980). Similarities in age of onset, time course and pathogenesis have suggested GSDM to be analogous to PPMS in humans. The genes present in the MHCII have been identified as having strong associations to multiple sclerosis in humans and several novel gene associations have been found PPMS (Hackenstein et al. 2001; Schmidt et al. 2002) and RRMS (Green et al. 2001; Ristić et al., 2005.). In order to better understand the genetics and the relationship between these 2 diseases, the following genetic studies have been performed.

Clinical signs and diagnosis of GSDM and PPMS

In GSDM there are no definitive diagnostic tests. Diagnosis of DM is made by clinical history. Clemmons describes history of progressive spinal ataxia and weakness that may have a waxing and waning course or be steadily progressive (Clemmons 1992). This is supported by the neurologic findings of a diffuse thoracolumbar (TL) spinal cord dysfunction. Diagnostic examinations are generally normal except for an elevated cerebral spinal fluid (CSF) protein in the lumbar cistern. Electromyographic (EMG) examination reveals no lower motor unit disease, supporting the localization of the disease process in the white matter pathways of the spinal cord.

Spinal cord evoked potentials recorded during the EMG do show changes which help determine the presence of spinal cord disease. Radiographs of the spinal column including myelography are normal (other than old age changes) in uncomplicated DM. MRI does not show specific changes, but there is speculation that myelography and MRI may show a diminished size of the thoracolumbar spinal cord due to spinal cord atrophy (Jones JC,et al. 2005). There are no laboratory tests, symptoms, or physical findings to diagnose PPMS. The MS diagnosis can only be made by a process of ruling out other possibilities. The diagnosing criteria has been established as such: 1) there must be evidence of two exacerbations, flare ups, or relapses defined clinically as the sudden appearance of an MS symptoms, which lasts more than 24 hours; 2) the exacerbations must be separated in temporally and spatially; and 3) there must be no other explanation for these exacerbations (Rolak 1996). Tests such as MRI, and evoked response (EP) testing have played an important role in the diagnostic process (Bashir and Whitaker 1999; Thompson, Montalban et al. 2000). The MRI is not always helpful in PPMS and normal MRI does not rule out a diagnosis of PPMS.

Etiology and Pathogenesis of GSDM and PPMS

In GSDM the etiology and pathogenesis are unknown. Although over the past three decades there has been new insight into the pathoetiology of GSDM. An immune-mediated pathogenesis of GSDM was suggested by Waxman et al. (Waxman, Clemmons et al. 1980; Waxman, Clemmons et al. 1980). This study shows a depression of T cell response to the mitogen, including concanavalin A and phytohemagglutinin P, were reported in peripheral blood of GSDM; and a presence of the activated suppressor cell in the peripheral blood was proposing to result in a depression of T cell response (Waxman, Clemmons et al. 1980; Waxman, Clemmons et al. 1980). The depression of their cell mediated immune responses correlates with the clinical stage and severity of the disease (Waxman, Clemmons et al. 1980) Furthermore,

this suppression has been shown to be due to the genesis of a circulating suppressor cell (Waxman, Clemmons et al. 1980). While the relationship to these findings and the onset of the clinical disease remains unclear, the evidence suggests that DM is secondary to the activation of an autoimmune lymphocyte population and this leads to a subsequent immune attack upon their own myelin. Antigen-binding cells specific to canine myelin basic protein have been demonstrated in some dogs with DM. Immunoglobulins have been shown to be bound within lesions in the spinal cords of dogs with GSDM (Barclay and Haines, 1994). Even as the cause of the altered immune system remains unknown, what is becoming increasingly clear is that GSDM is caused by an autoimmune disease attacking the nervous systems that leads to progressive neural tissue damage. In many aspects, GSDM is similar to the pathogenesis that has been discovered in MS in human beings.

Like GSDM exact etiology and pathogenesis of PPMS is unknown, several factors appear to be involved (Fischman 1982). MS has been described to be genetic in nature with underlying environmental factors playing a role in its progression. The current theory accepts MS to involve an autoimmune process directed at the central nervous system (Bitsch, Dressel et al. 2004).

Genetic Significance of GSDM Related to Multiple Sclerosis

A repeated genetic association has been made between MS and the Human leukocyte antigen (HLA) MHC class II- DRB1 gene, located on chromosome 6 in humans. Although the HLA-DRB1*1501-DQB1*0602 haplotype association has been made repeatedly in high risk northern European MS populations, [Oksenberg] reports a selective association with DRB1*15, indicating a primary role for the DRB1 locus in MS patients independent of DQB1 (Oksenberg *et al*. 2004).

The HVR regions of the Dog leukocyte antigen (DLA) MHC class II-DRB1 located on chromosome 12, the canine homolog to HLA-DRB1, have been studied in depth due to its highly

polymorphic nature and associations with other autoimmune disorders such as canine narcolepsy (Chabas et al. 2003) and rheumatoid arthritis (Ollier et al. 2001). The latest DLA nomenclature report list 52 DLA-DRB1 alleles (Kennedy *et al*. 2002). Within the DLA-DRB1 there are three HVR in which the polymorphisms are grouped, HVR 1 [amino acid (AA) position 8-16], HVR 2 (AA position 26-39) and HVR 3(AA position 57-74). These hypervariable regions allow for analysis of the DLA-DRB1.

Further genetic associations have been made in relationship to PPMS and RRMS. In addition to the MCH II, the chromosome 19q13 region surrounding the apolipoprotien E (APOE) gene has shown consistent evidence of involvement in MS when family-based analyses were conducted (Schmit et al. 2002). It has been shown by Schmit et al. that the APOE-4 allele has a greater association to more severe forms of MS to include PPMS. PPMS also shows an increased frequency of the R551 variant of IL4R as compared to RRMS patients (Hackstein et al. 2001). In contrast, a TGFB1 haplotype was found by Green et al. to have an association to a mild disease course represented by RRMS. A polymorphism within the HFE gene was found by Ristic et al. to be associated with forms of MS exhibiting early onset and mild course such as that in RRMS.

Purpose of Thesis Research

Based upon the hypothesis that GSDM has a genetic predisposition and closely related to PPMS, the purpose of this thesis was to further our understanding of the relationship between these diseases by evaluating the genetic similarities between GSDM and PPMS. To accomplish this, we analyzed various genetic markers associated with MS.

CHAPTER 2
ANALYSIS OF THE DLA-DRB1 IN GERMAN SHEPHERD DEGENERATIVE MYELOPATHY

Introduction

Degenerative Myelopathy of the German shepherd dog (GSDM) was first described in 1973 as a specific degenerative neurologic disease in the German shepherd dog (GSD) (Averill 1973). GSDM is characterized by wide spread myelin and axon loss in the thoracolumbar area of the spinal cord (Clemmons 1992). Beginning as a thoracolumbar spinal cord disease it progresses to involve the majority of the neural axis, with the most significant pathology remaining in the spinal cord. Although few cases have been reported in other large breed dogs, the disease appears with relative frequency in the German shepherd breed, suggesting that there is a genetic predisposition for GSDs in developing DM (Clemmons, web)[1]. GSDM has no specific etiology known to date. The most probable hypothesis is that DM represents an immune-related neurodegenerative disease (Clemmons 1992; Barclay and Haines 1994). The concentration of IgG in the cerebrospinal fluid from GSDs affected with GSDM is markedly increased, which can be demonstrated as oligoclonal bands by isoelectrofocusing (Kamishina H, et al.). This observation supports our hypothesis that GSDM shares pathoetiology with MS.

GSDM is chronic and progressive in nature and increasingly bears similarities to primary progressive multiple sclerosis (PPMS) in humans. Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system (CNS) characterized by myelin loss, varying degrees of axonal pathology, and progressive neurological dysfunction (Averill 1973; Goodkin, Doolittle et al. 1991). A repeated genetic association has been made to the Human leukocyte antigen (HLA) MHC class II- DRB1 gene (Brassat, Salemi et al. 2005; Prat, Tomaru et al. 2005).

[1] neuro.vetmed.ufl.edu

Within the dog leukocyte antigen (DLA)-DRB1 there are three HVRs in which the polymorphisms are grouped, HVR 1(amino acid (AA) position 8-16), HVR 2 (AA position 26-39) and HVR 3(AA position 57-74) (Francino, Amills et al. 1997). The hypervariable regions (HVRs) of the DLA- DRB1 have been studied in depth due to their highly polymorphic nature and associations with other autoimmune disorders such as rheumatoid arthritis (Ollier, Kennedy et al. 2001) and canine narcolepsy (Chabas, Taheri et al. 2003).

Through the analysis of the HVRs within the DLA-DRB1 region it was our goal to provide a genetic association to be made between the DLA-DRB1 gene and GSDM, an association analogous to that between HLA-DRB1 and MS in humans. To analyze the HVRs of the DLA-DRB1 in GSDM we performed PCR followed by direct sequencing of the DLA-DRB1.

Materials and Methods

Blood samples were obtained from 15 German Shepherd dogs already defined as GSDM at the neurology service of University of Florida Veterinary Medical Center and by donation from private parties. Samples were as follows: 6 GSDs presenting with GSDM, 5 GSD presenting with neurological disorder other than GSDM, to include cervical spondylomyelopathy and intervertebral disk disease (IVDD), and 4 healthy GSDs. Blood was collected in lavender top CBC tubes containing EDTA and stored at 4ºC until DNA isolation was preformed from the whole blood sample. DNA was isolated using the AquaPure Genomic Isolation kit (Bio-Rad).

A diagnosis of GSDM was confirmed by necropsy in 2 samples. The remaining 4 GSDM samples were clinically diagnosed as probable GSDM using our clinical criteria (see Clemmons' web for detail)[2]. These criteria are based on:

1. German Shepherd with appropriate clinical signs

[2] neuro.vetmed.ufl.edu

2. Normal electromyogram (EMG) with abnormal spinal evoked potential

3. Increase of total protein in cerebrospinal fluid (CSF) drawn from the lumbar cistern, along with an elevated cholinesterase level above 300 IU/ml

4. MRI or myelogram with negative results for compressive lesions of the spinal cord

Polymerase chain reaction (PCR) was performed to select for the DLA-DRB1 using the forward primer (5'-3')- TATCCCGTCTCTGCAGCACATTTC and reverse primer (5'-3') TGCCCGCTGCACACTGAAACTCTC. (Amills, Francino et al. 1995; Francino, Amills et al. 1997). The PCR reaction was performed using 100ng of isolated genomic DNA in a 50ul reaction which contained 1 X PCR Buffer with 1.5mM Mg2+, 0.5mM Mg2+ (final concentration of 2mM Mg2+), 0.1ul of forward and reverse primers (1uM final concentration), and 2 units of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturization at 95ºC followed by 94º C for 60 sec, 55ºC for 30 sec, 72ºC for 40 sec for 35cycles, followed by a final extension at 72ºC for 15min and a 4ºC hold. Amplified samples were viewed by running 5ul of PCR product on a 1.5% agarose gel containing 5ug ethidium bromide. The correct PCR product was cut from the gel and purified using Freeze-N-Squeeze Gel Purification Columns (Bio-Rad). The gel purified product was further purified with Stratagene Perfect Prep purification columns. Purified samples were brought to the concentration of 0.1ug/ul. Sequencing reactions were performed in both forward and reverse directions. All sequencing was performed by University of Florida DNA sequencing core/ICBR. Sequence analysis was performed using NCBI BLAST. BLAST was used to determine the most probable DLA- DRB1 allele present in homozygous samples. The two alleles present in the heterozygous samples could not be clearly defined due to the hypervariability of the DLA-DRB1 and the number of alleles

previously defined. Heterozygous samples were analyzed by heterozygous points within HVR2 and HVR3.

Results and Discussion

One normal GSD presented as homozygous with allele DLA-DRB1 *15. Fourteen of the 15 dogs were heterozygous and therefore the heterozygous patterns were analyzed. In GSDM, the HVR2 was homozygous throughout in 5 samples (Figure1) followed by two heterozygous points, one of which was within HVR3. All 5 GSDs with other neurological diseases had a heterozygous point in the HVR2. Similarly, 3 of the 4 healthy GSDs had a heterozygous point in this position. Based on the sequence patterns present in 5 of the GSDM samples it suggests that there is a relationship between the occurrence of GSDM and the DLA-DRB1 alleles present. This supports GSDM as having a genetic predisposition based on the MHC II analogous to MS. Further analysis of the DLA-DRB1 is needed to determine the exact alleles present in the heterozygous samples, along with a more in depth study of the DLA region.

The DLA region is composed of several genes in which the DLA-DRB1 alleles tend to occur in only one combination with DQA1 and DQB1 alleles (Kennedy et al. 2002). This is similar to the situation found for HLA class II in human populations (Ostrander, Galibert et al. 2000; Kennedy, Barnes et al. 2002). In following with the research performed for the analysis of the HLA haplotypes in MS, DLA-DQA1 and DLA-DQB1 analysis is currently being performed on GSDM samples. Data will be combined with that of the DLA-DRB1 in an attempt to determine DLA haplotypes that can be associated with GSDM.

Figures

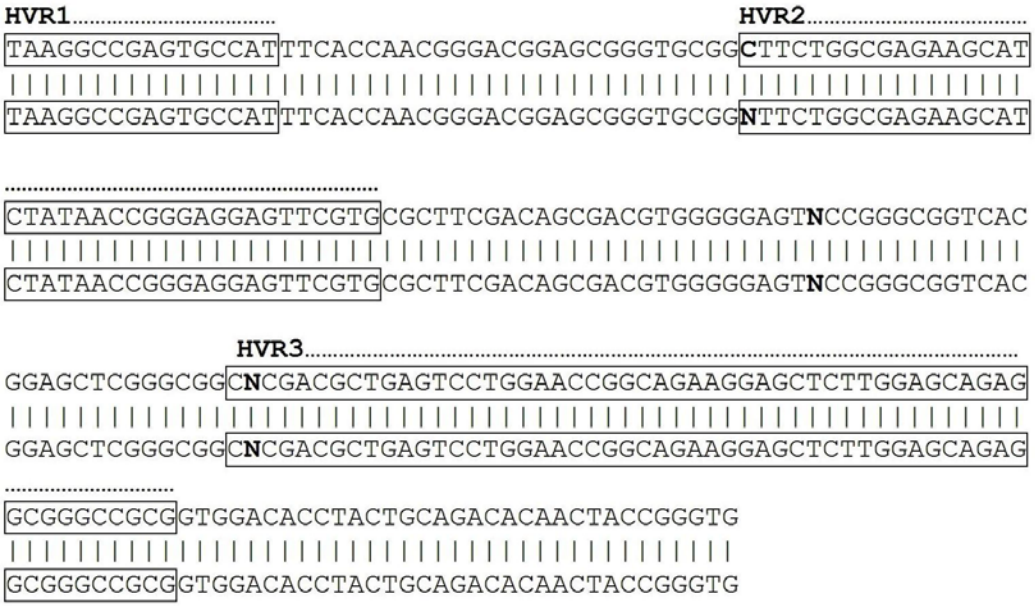

Figure 2-1. Comparison of the DLA-DRB1 sequences and HVRs between GSDM and normal GSDs or GSDs with other neurological diseases. Two sequences are lined to compare the two sequences and HVRs. The top row represents the sequence of GSDM and the bottom row represents that of normal GSD or GSD with neurological diseases other than GSDM. Boxed areas show 3 HVRs; HVR1 is located at nucleotide position 1-16, HVR2 44-62, and HVR3 143- 208. In GSDM samples, there is a consistent pattern of homozygousity throughout HVR2. The first set of nucleotides, position 44, in HVR2 in GSDM samples is homozygous whereas that of normal GSD or GSD with other neurological diseases is heterozygous. In all samples, there are heterozygous points at nucleotide position 108 and in HVR3 at nucleotide position 144.

CHAPTER 3
HAPLOTYPING OF THE DOG LEUKOCYTE ANTIGEN IN THE GERMAN SHEPHERD DOG

Introduction

Degenerative Myelopathy of the German shepherd dog (GSDM) is a specific disorder characterized by wide spread myelin and axon loss in the thorocoloumbar area of the spinal cord (Clemmons.1992). Although few cases have been reported in other large breed dogs, the disease appears with relative frequency only in the German shepherd breed, suggesting that there is a genetic predisposition for GSD in developing DM (Clemmons, web)[3]. GSDM has no specific etiology known to date. The most probable hypothesis is that DM represents an immune-related neurodegenerative disease (Clemmons. 1992). It is suspected that an altered immune system which attacks the nervous system is a major cause of GSDM.

GSDM is a devastating chronic progressive neurological disease present in the German shepherd breed that bares clinical similarities to multiple sclerosis (MS) in humans. MS is a common inflammatory disease of the central nervous system (CNS) characterized by myelin loss, varying degrees of axonal pathology, and progressive neurological dysfunction (Hauser and Goodkin, 2001). MS has been described to be genetic in nature with underlying environmental factors playing a role in its progression. MS is an autoimmune disease that has been repeatedly shown to be associated with the highly polymorphic HLA-DRB1 region of the MCH II complex. More specifically a HLA-DRB1, DBA, DQB haplotype has been found to be linked in family based studies. The HLA MHC class II- DRB1 gene is located on chromosome 6 in humans. Although the HLA-DRB1*1501-DQB1*0602 haplotype association has been made repeatedly high risk northern European MS population, [Oksenberg] reports a selective association with

[3] neuro.vetmed.ufl.edu

DRB1*15, indicating a primary role for the DRB1 locus in MS patients independent of DQB1 (Oksenberg *et al*. 2004). The HLA class II DR2 haplotype (DRB1*1501, DQA1*0102, DQB1*0602) has been associated in varying degrees with MS in all ethnic groups, but particularly strongly in Caucasians (Hillert 1994; Fernandez, Fernandez et al. 2004)

The HVR regions of the Dog leukocyte antigen (DLA) MHC class II-DRB1 located on chromosome 12, the canine homolog to HLA-DRB1, has been studied in depth due to its highly polymorphic nature and associations with other autoimmune disorders such as canine narcolepsy (Chabas et al. 2003) and rheumatoid arthritis (Ollier et al. 2001). The DLA region is composed of several genes in which the DLA-DRB1 alleles tend to occur in only one combination with DQA1 and DQB1 alleles. DLA-DQ alleles, especially DQA1 alleles, appear to occur in combination with multiple DLA-DRB1 types. This is similar to the situation found for HLA class II in human populations (Ostrander, Galibert et al. 2000; Kennedy, Barnes et al. 2002).

Based on the commonalties of the diseases, the DLA-DRB1, DQA, DQB haplotype of 122 German Shepherd dogs (GSD) was analyzed to determine if there was a predominant haplotype present that could be directly associated to GSDs presenting with DM. This was accomplished using PCR to amplify the DLA-DRB1, DQA and DQB region, followed by direct sequencing of 15 control samples. Haplotyping of remaining 107 samples was performed using denaturing gradient gel electrophorisis (DGGE). Haplotyping revealed data consistant with previously described results in the GSD, but no one haplotype associated with GSDM was found.

Materials and Methods

Collection of Samples

Blood samples were obtained from dogs throughout the US by provided by contacts within the GSDCA (German Shepherd Dog Club of America). Basic information about the subjects was

collected pertaining to history of neurologic disease and family history of neurological disease if known.

Blood was collected in lavender top CBC tube containing EDTA from which 200ul was placed onto a FTA DNA card (Whatman). Local veterinary technicians collected the blood, labeled and mailed in the samples. Once the sample was received on the FTA card DNA isolation was preformed from the whole blood sample per manufactures instructions. DNA isolated on FTA disc was used for downstream PCR reaction.

DNA Amplification

DLA-DRB1

Polymerase chain reaction (PCR) was performed to select for the DLA-DRB1 using the forward primer (5 ’-3’) - CCGTCCCCACAGCACATTTC and reverse primer (5’-3’) TGTGTCACACACCTCAGCACC (Angles et al. 2005). The PCR gave an amplified product containing several bands. The correct product was selected based on the expected size of the DLA-DRB1 region amplified ~270bp.

The PCR reaction was performed using one FTA disc containing isolated and cleaned genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 0.1ul of forward and reverse primers (1uM final concentration) and 2 unit of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturazation at 95`C followed by 94`C for 30 sec, 57`C for 30 sec, 72`C for 30 sec for 35cycles, followed by a final extension at 72`C for 15min and a 4`C hold. Reactions took place in either an Icycler or Mycycler (Bio-Rad) based on availability. Amplified samples were viewed by running 5ul of PCR product on a 1.5% agarose gel containing 5ug ethidium bromide.

DLA-DQA

Polymerase chain reaction (PCR) was performed to select for the DLA-DQA using the forward primer (5 ’-3’) - TAAGGTTCTTTTCTCCCTCT and reverse primer (5’-3’)

GGACAGATTCAGTGAAGAGA (Angles et al. 2005). The PCR gave an amplified product of ~346bp

The PCR reaction was performed using one FTA disc containing isolated and cleaned genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 0.1ul of forward and reverse primers (1uM final concentration) and 2 unit of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturazation at 95`C followed by 94`C for 30 sec, 57`C for 30 sec, 72`C for 30 sec for 35cycles, followed by a final extension at 72`C for 15min and a 4`C hold. Reactions took place in either an Icycler or Mycycler (Bio-Rad) based on availability. Amplified samples were viewed by running 5ul of PCR product on a 1.5% agarose gel containing 5ug ethidium bromide.

DLA-DQB

Polymerase chain reaction (PCR) was performed to select for the DLA-DQB using the forward primer (5 ’-3’)- TCACTGGCCCGGCTGTCTCC and reverse primer (5’-3’) GGTGCGCTCACCTCGCCGCT (Angles et al. 2005). The PCR gave an amplified product of ~267bp.

The PCR reaction was performed using one FTA disc containing isolated and cleaned genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 0.1ul of forward and reverse primers (1uM final concentration) and 2 unit of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturazation at 95`C followed by 94`C for 30 sec, 60`C for 30 sec, 72`C for 30 sec for 35cycles, followed by a final extension at 72`C for 15min and a 4`C hold. Reactions took place in either an Icycler or Mycycler (Bio-Rad) based on availability. Amplified samples were viewed by running 5ul of PCR product on a 1.5% agarose gel containing 5ug ethidium bromide.

DNA Sequencing

The correct PCR product was cut from the gel and purified using Freeze-N-Squeeze Gel Purification Columns (Bio-Rad). The gel purified product was further purified with Stratagene Perfect Prep purification columns. Purified samples were brought to the concentration of 0.1ug/ul. Sequencing reactions were performed in both forward and reverse directions. All sequencing was performed in an outside lab.

Sequence Analysis

Sequence analysis was performed using NCBI BLAST. BLAST was used to determine the allele present in homozygous samples. These homozygous alleles were used as controls.

Denaturing Gradient Gel Electrophoresis

PCR was performed to add GC a clamp to the selected regions to be analyzed. The primers and conditions are as followed:

DQA, 5' GC clamp

CGCCCCGCCGCGCCCCGCGGCCCGGTCCCCGCGCTAAGGTTCTTTTCTCTCCCTCT

The PCR reaction was performed using one FTA disc containing isolated and cleaned genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 2.5ul of forward and reverse primers (2.5uM final concentration), and 2 unit of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturazation at 95`C followed by 94`C for 30 sec, 60.5`C for 30 sec, 72`C for 30 sec for 40cycles, followed by a final extension at 72`C for 15min and a 4`C hold. Reactions took place the Icycler (BioRad). Amplified samples were viewed by running 5ul of PCR product on a 1.5% agarose gel containing 5ug ethidium bromide.

DRB1 3’ GC clamp

TGTTGCACACACCTCAGCACCACGCGCCCCTGGCCCGCGCGCCCCGCGCCGCCCCGC

The PCR reaction was performed using one FTA disc containing isolated and cleaned genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 2.5ul of forward and reverse primers (2.5uM final concentration),and 2 unit of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturazation at 95`C followed by 94`C for 30 sec, 50`C for 30 sec, 72`C for 30 sec for 40 cycles, followed by a final extension at 72`C for 15min and a 4`C hold. Reactions took place the Icycler (BioRad).

Amplified samples were viewed by running 5ul of PCR product on a 1.5% agarose gel containing 5ug ethidium bromide.

DQA 3’ GC clamp

GGTGCGCTCACCTCGCCGCTCGCGCCCCTGGCCCGCGCGCCCCGCGCCGCCCC GC

The PCR reaction was performed using one FTA disc containing isolated and cleaned genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 2.5ul of forward and reverse primers (2.5uM final concentration),and 2 unit of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturazation at 95`C followed by 94`C for 30 sec, 50`C for 30 sec, 72`C for 30 sec for 40cycles, followed by a final extension at 72`C for 15min and a 4`C hold. Reactions took place in the Icycler (BioRad). Amplified samples were viewed by running 5ul of PCR product on a 1.5% agarose gel containing 5ug ethidium bromide.

Homozygous controls determined by direct sequencing for DRB1, DQA and DQB were run on a 0%to 75% denaturing gradient gel at 60`C for 4hrs at 120 volts. Gels were stained with

syber green and viewed on the GelDoc (BioRad) for banding patterns and band position determination. Subsequent samples were run under the same conditions and compared against controls for allele determination based on band location.

Results and Discussion

There was no correlation found between the DLA haplotype in GSDs presenting with DM in the current study. Although, there may be a link to a DLA haplotype a more detailed pedigree history with definitive GSDM in pedigree would be required. Haplotyping confirmed the results of recently published data on the relationship of the DLA alleles within a particular breed. Kennedy et al. reports extensive interbreed variation with minimal intrabreed variation (Kennedy et al. 2002). The GSD has been found to present with 7 DRB1, 5 DQA and 8 DQB (Angles et al. 2005). Of the previously described alleles in the GSD, in this study we found 3 DRB1, 4 DQA and 3 DQB: DRB1*1011, *1501and *1502, DQA *601,*401,*201 and *101 and DQB *2301, *1302 and *301. There were no new alleles found. Due to the limited number of homozygous controls only 32 samples were fully haplotyped.

Table 3-1. DLA allele frequencies for 32

DRB1	# Detected	Frequency
1011	4	.125
1501	7	.219
1502	4	.125
1101/1501	8	.250
1101/1502	9	.281

DQA	# Detected	Frequency
101	0	0
201	4	.125
601	12	.375
201/101	7	.219
201/401	1	.031
201/601	7	.219
101/601	1	.031

DQB	# Detected	Frequency
301	10	.313
1302	4	.125
2302	8	.250
301/1302	10	.313

Table 3-2. DLA haplotype frequency for 32 GSDs

Haplotype DRB1-DQA-DQB	# Detected	Frequency
1011-201-1302	2	.063
1011-601-1302	1	.031
1501-201-301	1	.031
1501-601-301	6	.188
1502-601-301	3	.094
1502-601-1302	1	.031
1101-201-301/1302	1	.031
1011/1501-201/601-301/1302	3	.094
1011/1501-601-23*	1	.031
1101/1501-601/101-301/1302	1	.031
1011/1501-201/101-*23	1	.031
1101/1502-201/101-301/1302	1	.031
1011/1502-201/401-301/1302	1	.031
1011/1502-201/101-23*	5	.156
1011/1502-201/601-23*	1	.031

CHAPTER 4
ANALYSIS OF MBP IN GERMAN SHEPHERD DEGENERATIVE MYELOPATHY

Introduction

Degenerative Myelopathy of the German shepherd dog (GSDM) is a specific disorder characterized by wide spread myelin and axon loss in the thoracolumbar area of the spinal cord (Clemmons.1992). One of the most striking features is that of decreased myelin in the white matter of the spinal cord (Clemmons 1992). Destruction of the myelin sheath is one of the pathological hallmarks of German shepherd degenerative myelopathy and has been hypothesized as a result of an autoimmune attack on the central nervous system.

Like GSDM, Multiple sclerosis (MS) is a chronic inflammatory disease characterized by multifocal damage in the central nervous system myelin (Tienari et al., 1997). Autoimmunity against myelin basic protein (MBP) or other components of the myelin sheath has been proposed as a pathogenetic mechanism leading to demyelination (Wucherpfennig et al., 1991; Voskuhl et al., 1993; Kerlero de Rosbo et al., 1993; Raine, 1997; Tienari et al., 1997). HLA-association (Olerup and Hillert, 1991) studies have established the concept that genetic factors contribute to the expression of the disease (Tienari et al., 1997). Evidence has been found for the contribution of three loci in determining MS-susceptibility (Tienari et al., 1992a,1993; Kuokkanen et al., 1996., namely a locus linked to the MBP gene on chromosome 18, a locus close to or within the HLA-DRrDQ subregion on chromosome 6, and a locus on chromosome 5p14–p12 (Tienari et al., 1997).

In the present study, through the analysis of the MBP gene it was our goal to provide a genetic association to be made between MBP and GSDM, an association analogous to that between MBP and MS in humans. To analyze the MBP gene in GSDM we performed PCR followed by agarose gel analysis to determine band size and banding patterns present.

Materials and Methods

Sample Collection

Blood samples were obtained from 33 German shepherd dogs seen at the neurology service of University of Florida Veterinary Medical Center and by donation from private parties. Samples were as follows: 24 GSDs presenting with GSDM and 19 healthy GSDs. Blood was collected in lavender top CBC tubes containing EDTA and stored at 4ºC until DNA isolation was preformed from the whole blood sample. DNA was isolated using the AquaPure Genomic Isolation kit (Bio-Rad). A diagnosis of GSDM was confirmed by necropsy in 2 samples. The remaining 22 GSDM samples were clinically diagnosed as probable GSDM using our clinical criteria (see Clemmons' web for detail)[4]. These criteria are based on:

1. German Shepherd with appropriate clinical signs

2. Normal electromyogram (EMG) with abnormal spinal evoked potential

3. Increase of total protein in cerebrospinal fluid (CSF) drawn from the lumbar cistern, along with an elevated cholinesterase level above 300 IU/ml

4. MRI or myelogram with negative results for compressive lesions of the spinal cord

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed to select for a 450bp product from the MBP gene using the forward primer (5 '-3') – ATATGTGGATGGATGGATGACGAAT and reverse primer (5'-3') CAGGATTCACTCACATATTCCTG (Invitrogen).

The PCR reaction was performed using 100ng of isolated genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 0.25ul of forward and reverse primers (2.5uM final concentration),and 2 unit of Taq Polymerase (Eppendorf). The

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PCR program started with an initial 5 min denaturazation at 95`C followed by 95`C for 60 sec, 58`C for 60 sec, 72`C for 60 sec for 35cycles, followed by a final extension at 72`C for 5min and a 4`C hold.

Imaging and Analysis

All amplified samples were viewed by running 10ul of PCR product on a 2% agarose gel containing 5ug ethidium bromide. Gels were imaged using a GelDoc molecular imager (BioRad). Analysis of product was performed using Quantity One (BioRad). PCR products were analyzed for variation in size, intensity and banding patterns.

Results and Discussion

In this study, we found size variation in the segment of the MBP allele amplified. A product of ~350bp was found in 88% of dogs with GSDM and only in 57% of healthy GSDs. This disparity in product size is suggestive of a 70 bp tandem repeat (TGGA) deletion that correlates to the same deletion found in a population of MS patients in Finland, Pihlaja et al.

In previous studies, the HLA-DRB1 has consistently been found to be associated with MS. Other candidate gene studies have focused components on myelin, such as MBP (Guerini et al., 2003; Sudomoina and Favorova, 2003; Oskenberg et al., 2001). The findings of this study show the polymorphism of MBP appears to contribute to the etiology for HLA-DR4 and HLA-DR5 positive Italians and Russians (Guerini et al., 2003).

In GSDM analysis of DLA-DRB1 showed dogs presenting with DM to be homozygous for DLA-DRB1*1101 and revealed a homozygous point in HVR2 of the DLA-DRB1 in the heterozygous samples presenting with only one copy of DLA-DRB1*1101 (Clemmons 2006). Additional studies into the correlation between MBP and specific DLA alleles need to be conducted to further understand the genetic relationship in allelic association GSDM as was done in human studies.

The findings of this study suggest that two diseases may share not only pathologic features, but also genetic factors involved in their pathogenesis. Together with the finding on the MHC II region, it is thought that autoimmune responses targeted to the CNS tissues including MBP plays an important role in the development of GSDM.

Figures

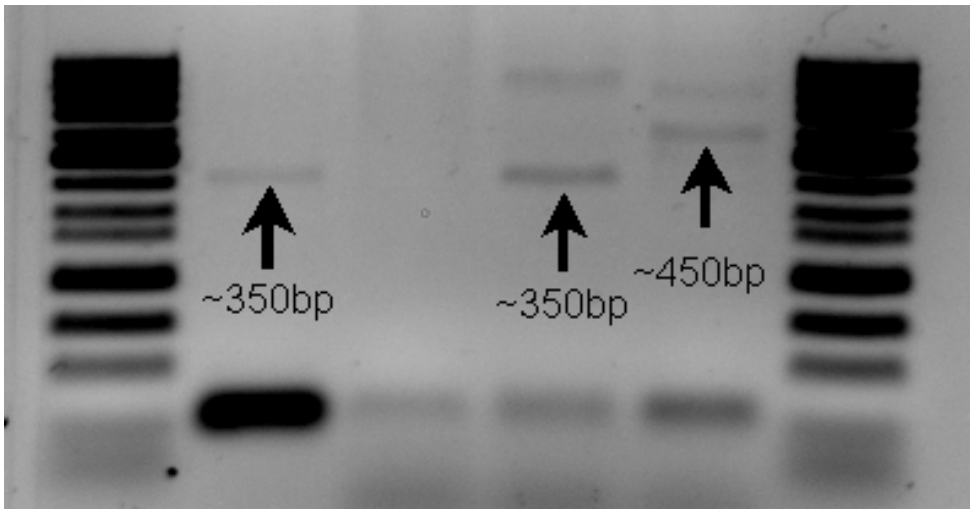

Figure 4-1. PCR of MBP region of canine genome in GSDM patients (1, 3, and 4) showing possible 70 bp deletion in patient 1 and 3.

CHAPTER 5
GENETIC ANALYSIS OF GERMAN SHEPHERD DEGENERATIVE MYELOPATHY AS COMPARED TO MAJOR FORMS OF MULTIPLE SCLEROSIS

Introduction

Degenerative Myelopathy of the German shepherd dog (GSDM), a devastating chronic progressive neurological disease present in the German shepherd breed, bares clinical similarities to multiple sclerosis (MS) in humans. MS is a common inflammatory disease of the central nervous system (CNS) characterized by myelin loss, varying degrees of axonal pathology, and progressive neurological dysfunction (Hauser and Goodkin, 2001). MS has been described to be genetic in nature with underlying environmental factors playing a role in its progression. MS is described in three categories based on the time of onset, severity and disease progression; 1) Relapsing remitting (RRMS), 2) Secondary progressive (SPMS) and 3) Primary progressive (PPMS).

In MS in general, repeated genetic association has been made to the Human leukocyte antigen (HLA) MHC class II- DRB1 (Oksenberg et al. 2004). Further analysis of the individual categories of MS have yielded genetic association in various additional genes to include apolipoprotein E (APOE), interleukin-4 receptor (IL4R), hemochromatosis gene (HFE) and transforming growth factor beta-1 (TGFB1).

One of the first non-MHC regions of interest identified through linkage analysis was chromosome 19q13 near Apolipoprotien E (APOE [MIM 107741]) locus (Haines et al. 1993, 1996; Schmidt et al.). The apoE protein has long been associated with the regeneration of axons and myelin after lesions of central and peripheral nervous tissue (Ignatius et al. 1986; Schmit et al. 2002). Its isoforms have been shown to have differential effects on neural growth (Nathan et al. 1994; Schmit et al. 2002). Apolipoprotien E (APOE) gene has shown consistent evidence of involvement in MS when family-based analyses were conducted. Furthermore, several clinical

reports have suggested that APOE-4 allele may be associated with more severe disease and faster progression of disability (Schmit et al. 2002).

Like APOE, IL4R presents with various isoforms, one of which has been shown to maybe influence the genetic predisposition for PPMS (Hackstien et al. 2001). In experimental allergenic encephalomyelitis (EAE), an animal model of inflammatory demyelination, Th2 cells dominate the inflammatory response during the remission state. These cells are generated under interleukin -4 (IL-4). This cytokine is believed to play a beneficial role in the pathogenesis of MS by propagating anti-inflammatory mechanisms. The course of EAE is ameliorated by IL-4 (Racke et al. 1994; Shaw et al. 1997; Hackstein et al. 2001). A recent linkage and association analysis of certain candidate genes revealed a possible linkage of MS with marker D16S420 (43.2 cM) flanking, among others interleukin-4 receptor (IL4R) gene (He et al. 1998; Hackstein et al. 2001). Both genetic and functional data support the hypothesis that certain IL4R genotypes are strong candidates for MS susceptibility factors (Hackstein et al. 2001). PPMS patients were found to carry IL4R variant R551 more frequently than 305 healthy control subjects ($p=0.001$ for genotype differences; $P=101$ for allele frequency differences) (Hackstein et al. 2001).

Similar to PPMS, there are various genes associated with RRMS. There has been a consistency with linkage at ch.19q13 in MS genetic studies suggesting MS genes in this region. One such gene is the transforming growth factor-β1 gene (TGF-β1). TFG- β1, a pleieotropic cytokine with a significant role in the regulation of the immune homeostasis, serves as a potent chemoattractant in the early stages of inflammatory response, and as an immunosuppressant during resolution of such an event (Letterio and Roberts, 1997; Green at al., 2001). Seven TFGB1 polymorphisms have been identified (Cambein et al., 1996; Green et al., 2001). Distinct clinical phenotypes were examined and an association between a TFGB1 haplotype and mild

disease course was present (Green et al., 2001). RRMS has been associated with milder progression and earlier onset.

In the search for genes related to the genetic predisposition of MS it is often found that there is no association to susceptibility but to disease progression, as with the case of the hemochromatosis gene (HFE). Hemochromatatosis (HC) is a hereditary iron overload disorder. Two common disease mutations have been detected in the HFE gene (Ristić et al., 2005), H63D and C282Y. C282Y homozygosity confers the highest risk for iron overload (Ristić et al., 2005). Iron metabolism has been shown by Valberg et al. to play a role in MS. It was observed that patients carrying the mutant C282Y exhibited an earlier onset of disease symptoms (Ristić et al., 2005). The allele frequency of C282Y was measured in PPMS, SPMS and RRMS. There was a 0% allele frequency found in the 25 PPMS patients tested. Where as in RRMS there was a 3.70% allele frequency noted in 162 patients tested. Ristić et al suggest C282Y to be associated with forms of MS reminiscent of RRMS that have an early onset and slower progression.

While there is clinical evidence suggesting GSDM to be analogous to MS there has been no genetic evidence to support these findings. The purpose of this study was to evaluate the genetic similarities between GSDM and MS while differentiating genetically between the two major forms of MS, PPMS and RRMS.

Materials and Methods

Sample Collection

Blood samples were obtained from 12 German shepherd dogs seen at the neurology service of University of Florida Veterinary Medical Center and by donation from private parties. Samples were as follows: 6 GSDs presenting with GSDM and 6 healthy GSDs. Blood was collected in lavender top CBC tubes containing EDTA and stored at 4ºC until DNA isolation was preformed from the whole blood sample. DNA was isolated using the AquaPure Genomic

Isolation kit (Bio-Rad). A diagnosis of GSDM was confirmed by necropsy in 2 samples. The remaining 4 GSDM samples were clinically diagnosed as probable GSDM using our clinical criteria (see Clemmons' web for detail).[5] These criteria are based on:

1. German Shepherd with appropriate clinical signs

2. Normal electromyogram (EMG) with abnormal spinal evoked potential

3. Increase of total protein in cerebrospinal fluid (CSF) drawn from the lumbar cistern, along with an elevated cholinesterase level above 300 IU/ml

4. MRI or myelogram with negative results for compressive lesions of the spinal cord

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed to select for the APOE, IL4R, TGFB1 and HFE.

APOE

Forward primer (5'-3')- CGCTGGGTGCAGACACTGT and reverse primer (5'-3') GGCCTTCAACTCCTTCATGGT (UniSTS:273705) were used. The PCR reaction was performed using 100ng of isolated genomic DNA in a 50ul reaction which contained 1 X PCR Buffer with 1.5mM Mg2+,0.5mM Mg2+ (final concentration of 2mM Mg2+), 0.5ul of forward and reverse primers (1uM final concentration), and 2 units of Taq Polymerase (Eppendorf). The PCR protocol is as follows: initial 5 min denaturization at 95ºC followed by 95º C for 40 sec, 58ºC for 40 sec, 72ºC for 40 sec for 35cycles, followed by a final extension at 72ºC for 5min and a 4ºC hold.

IL4R

Forward primer (5'-3')- CGCTGGGTGCAGACACTGT and reverse primer (5'-3') GGCCTTCAACTCCTTCATGGT were used. The PCR reaction was performed using 100ng of

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isolated genomic DNA in a 50ul reaction which contained 1 X PCR Buffer with 1.5mM Mg2+,0.5mM Mg2+ (final concentration of 2mM Mg2+), 0.1ul of forward and reverse primers (1uM final concentration), and 2 units of Taq Polymerase (Eppendorf). The PCR protocol is as follows: initial 5 min denaturization at 95ºC followed by 95º C for 40 sec, 58ºC for 40 sec, 72ºC for 40 sec for 35cycles, followed by a final extension at 72ºC for 5min and a 4ºC hold.

TGFB1

Forward primer (5'-3')- AGTTGTTTTGTGTCGTTTCTGATGT and reverse primer (5'-3') GGCTAGCAGGTCTAGTAAAGATGG were used. The PCR reaction was performed using 100ng of isolated genomic DNA in a 50ul reaction which contained 1 X PCR Buffer with 1.5mM Mg2+,0.5mM Mg2+ (final concentration of 2mM Mg2+), 0.1ul of forward and reverse primers (1uM final concentration), and 2 units of Taq Polymerase (Eppendorf). The PCR protocol is as follows: initial 5 min denaturization at 95ºC followed by 95º C for 40 sec, 55ºC for 40 sec, 72ºC for 40 sec for 35cycles, followed by a final extension at 72ºC for 5min and a 4ºC hold.

HFE

Forward primer (5'-3')- CTCTCTTCTTAGTCTTGTTCCCAAA and reverse primer (5'-3') CTGCCTTCTGTGTCTCATGAATAA. The PCR reaction was performed using 100ng of isolated genomic DNA in a 50ul reaction which contained 1 X PCR Buffer with 1.5mM Mg2+,0.5mM Mg2+ (final concentration of 2mM Mg2+), 0.1ul of forward and reverse primers (1uM final concentration), and 2 units of Taq Polymerase (Eppendorf). The PCR protocol is as follows: initial 5 min denaturization at 95ºC followed by 95º C for 40 sec, 55ºC for 40 sec, 72ºC for 40 sec for 35cycles, followed by a final extension at 72ºC for 5min and a 4ºC hold.

Imaging and Analysis

All amplified samples were viewed by running 5ul of PCR product on a 2% agarose gel containing 5ug ethidium bromide. Gels were imaged using a GelDoc molecular imager

(BioRad). Analysis of product was performed using Quantity One (BioRad). PCR products were analyzed for variation in size, intensity and banding patterns.

Results and Discussion

Variations within the products of APOE and IL4R were noted; no variation within TGFB1 or HFE was visualized. Upon visualization of APOE there was an observable banding variation seen with the appearance of 2 bands one approximately 200bp and the second the expected 421bp band in 4 GSDM patients and a single band of 200bp or the expected 420bp band present in normal samples. IL4R revealed various banding patterns within GSDM patients and normal patients. GSDM patients predominantly appeared to have a 250bp band along with the 114bp band with normal controls having an 114bp band present. This band size dissimilarity is indicative of possible isoform diversity. The sequence differences in the isoforms are cause for priming error during the PCR process leading to varied product size.

The data presented suggests that, based on DNA variation, GSDM not only has a genetic basis but may be comparable to PPMS. The occurrence of two band sizes present in the analysis of APOE in the GSD suggests the presence of different isoforms. This is consistent with the study, Schmidt et al., of APOE in which APOE-4 and APOE-2 isoforms were found to have a significant association between a more severe case of MS and mild disease respectively. Further analysis into canine APOE isoforms is needed to confirm the isoform present in GSDM.

As with APOE, IL4R has isoforms that do not represent a general genetic risk factor to MS (Hackstien et al., 2001). It is suggested, Hackstien et al., that the R551 isoform may influence the genetic predisposition for PPMS. Further analysis into canine IL4R isoforms is needed to confirm the isoform present in GSDM.

The markers TGFB1 and HFE that were found to be associated with RRMS were found to have no variation in GSDM. In the study of TGFB1, Green et al., the only association found was

to a mild disease course comparable to that of RRMS. RRMS was found to have an association with a mutation within the HFE. According to Ristić et al., MS patients with the mutant C282Y allele exhibited earlier onset.

Based on the variations seen in APOE and IL4R and the lack of variation of TGFB1 and HFE it is suggested that GSDM has a similar genetic profile to PPMS.

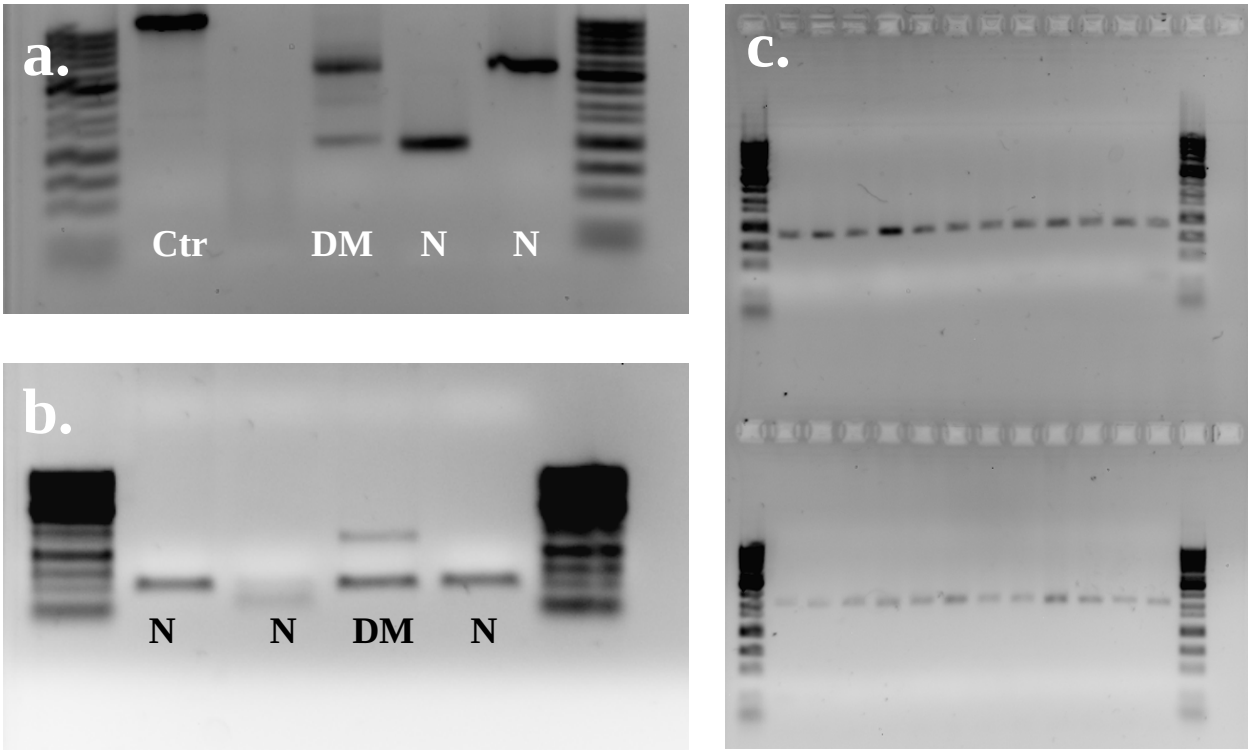

Figure 5-1. PCR of APOE (a.) and IL4R (b.) indicating differences in GSDM patients compared with controls. No differences were seen in PCR of HFE, TGFB.

CHAPTER 6
ANALYSIS OF THE SOD1 IN GERMAN SHEPHERD DEGENERATIVE MYELOPATHY

Introduction

Degenerative Mylopathy (DM) is a devastating chronic progressive neurodegenerative disease present in the German shepherd breed. The German shepherd breed has a relatively high incidence of DM suggesting heredity as a factor. GSDM is a specific disorder characterized by wide spread myelin and axon loss in the thoracolumbar area of the spinal cord (Clemmons.1992). Although few cases have been reported in other large breed dogs, the disease appears with relative frequency only in the German shepherd breed, suggesting that there is a genetic predisposition for GSD in developing DM (Clemmons, web)[6] GSDM has no specific etiology known to date.

GSDM is chronic and progressive in nature and bears similarities to Amyotrophic lateral sclerosis (ALS) in humans in that the spinal cord is affected. ALS is a progressive neurodegenerative disorder involving motor neurons in motor cortex, brain stem and spinal cord (Battistini et al, 2005). ALS has been described to be genetic in nature with cellular oxidative defenses playing a role in its progression. A clear genetic link has been made to mutations in the Cu/Zn superoxide dimutase (SOD1) gene of ALS patients. A repeated genetic association has been made to mutations in Cu/Zn superoxide dimutase SOD1. SOD1 is a cytostolic enzyme that catalyzes the conversion of the superoxide free radical (O2) into O2 and H_2O in eukaryotic cells (Green et al, 2002; Bannister et al.1991; Fridovich 1986a). Approximately 20% of all familial amyotrophic lateral sclerosis (FALS) cases and 2% of overall cases have identifiable mutations in this gene (Battistini et al, 2005).

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SOD1 is a highly conserved domain between species. Based on the commonalties of these diseases and the conservatism of the SOD1 gene 10 German Shepherd dogs (GSD) were evaluated for mutations in the SOD1 gene to determine if there was a predominant change within the region that could be directly associated to GSD presenting with DM. This was accomplished using PCR to amplify the SOD1 gene, followed by mutation detection using denaturing gradient gel electrophoresis (DGGE).

Materials and Methods

Sample Collection

Blood samples were obtained from 10 German shepherd dogs seen at University of Florida Veterinary Teaching Hospital. Samples were as follows: 8 GSDs presenting with DM, 1 GSD presenting with a neurological disorder other than DM and 1 normal healthy GSD. Blood was collected in lavender top CBC tube containing EDTA from which 200ul was placed onto a FTA DNA card (Whatman). Once sample was dry on the FTA card DNA isolation was preformed from the whole blood sample per manufactures instructions. DNA isolated on FTA disc was used for downstream PCR reaction.

DNA Amplification

Polymerase chain reaction (PCR) was performed to select for a 921bp product from the SOD1 gene using the forward primer (5 ’-3’) - GTGCAGGTCCTCACTTCAATC and reverse primer (5’-3’) ATCTTTGCCAGCAGTCACATT (Invitrogen). The PCR gave an amplified product of the correct expected size, approximately 921bp.

The PCR reaction was performed using one FTA disc containing isolated and cleaned genomic DNA in a 50ul reaction. The reaction included 1 X PCR Buffer with self adjusting Mg2+ (Eppendorf), 0.1ul of forward and reverse primers (1uM final concentration),and 2 unit of Taq Polymerase (Eppendorf). The PCR program started with an initial 5 min denaturazation at 95`C

followed by 94`C for 30 sec, 55`C for 30 sec, 72`C for 30 sec for 35cycles, followed by a final extension at 72`C for 15min and a 4`C hold. Amplified samples were viewed by running 10ul of PCR product on a 2% agarose E-gel (Invitrogen) containing ethidium bromide then visualized using a GelDoc (BioRad) molecular imager.

DGGE Mutation Detection

To further analyze the SOD1 region amplified denaturing gradient gel electrophoresis (DGGE) was performed PCR product was further analyzed using DGGE. 10ul of PCR product was run on an 8% polyacrylamide gel with a 0% to 100% denaturing gradient at 130V for 2 hrs. The gel was then stained with Sybr Green for visualization with a GelDoc (BioRad) molecular imager.

Results and Discussion

There were no visible banding differences seen on the 2% agarose gel. Further analysis to determine a possible mutation within the DNA sequence yielded no differential results. DGGE revealed no detectable change unique to GSD with clinically diagnosed DM.

SOD1 continues to be an important gene in neurodegenerative disease. DM has been reported in a smaller breed dogs, in particular the Pembroke Welsh Corgi. O'Brien et al, 2009 evaluated SOD1 immunoreactivity in the spinal neurons in dogs affected with DM and suggested the upper motor neuron form of ALS to be similar histologically and clinically to DM in the Pembroke Welsh Corgi. In the advanced stages, lower motor neuron involvement becomes apparent and ultimately progresses to bulbar signs, thus suggesting a phenotype similar to ALS (O'Brien et al, 2009). It was demonstrated that a mutation in the canine ortholog of *SOD1*gene (a gene commonly associated with familial ALS) is a significant risk factor for DM in the dog (O'Brien et al, 2009). Although, researchers at the University of Missouri have suggested that DM may be a form of amyotrophic lateral sclerosis (ALS) because of the association of SOD1

with motor unit diseases; data from the GSD patients do not support this. Although GSDM and ALS are both neurological in nature there appears to be strong differences in the two diseases. Amyotrophic lateral sclerosis (ALS) is an adult-onset, progressive paralysis of humans characterized by loss of motor neurons and sclerosis of the lateral funiculus (O'Brien et al, 2009); Whereas GSDM, though chronic and progressive is not characterized by loss of motor neurons. It is possible a change in SOD1 may trigger an immune attack upon the CNS supporting GSDM as an autoimmune disease. Corgis Myelopathy may be representative of ALS while GSDM is representative of PPMS.

Figures

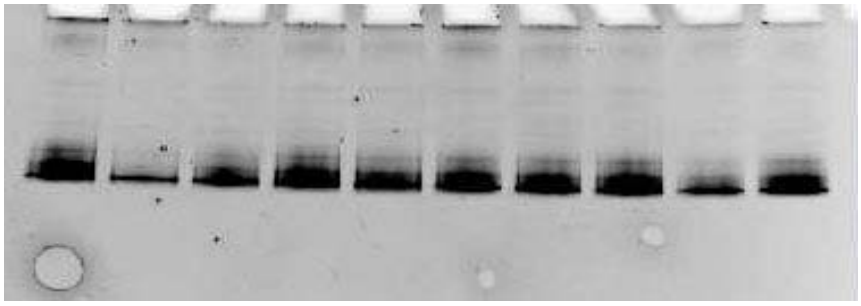

Figure 6-1. SOD1 run on a 0% to 100% denaturing gel showed no sign of mutation based on conformational changes within the gel run.

CHAPTER 7
LIMITATIONS IN THE STUDY AND CONCLUSION

Limitations

In this study, genetic markers for MS and ALS were evaluated. The results were adequately reproducible. In chapter 2, analysis of the DLA-DRB1 was performed. We demonstrated an allelic association based on variations within the HVR. In chapter 3, analysis of APOE, IL4R, HFE, and TGFB1 was performed. We showed a change in those genes related to PPMS (APOE and IL4R) and no change in those related to RRMS (HFE, TGFB1). In chapter 4, analysis of SOD1 a marker for ALS was performed. We showed no visible mutation within the gene and therefore no association to GSDM was noted.

Limitation in Sampling Groups

Numerous limitations were apparent. The first limitation is in samples of GSDM. The samples were obtained from clinically diagnosed patients. There was no pathological confirmation of GSDM performed in this study except in limited cases. In order to evaluate the full scope of genetic association in GSDM diagnosis and pathological confirmation of GSDM would be required in future studies. Second, control samples were obtained from clinical patients presented at the neurology service in this research. Clinical history showed various neurological disorders. It is possible that non-GSDM patients might still carry potential to develop GSDM at a future date. Furthermore, these were owned animals where necropsies were not always available even if requested.

The number of GSDM samples available for this study was small. A larger sample size of dogs presenting with GSDM would have provided a better means for more reliable statistical measurement. Comparing the results for GSDM patients with other neurologic diseases, both

inflammatory and non-inflammatory in nature, left questions as to if there could be an association of the genes to other neurological disorders.

Limitation with DLA-DRB1 Allele Determination

DLA-DRB1 gene product was verified by direct sequencing of the PCR product. Although we were able to verify the correct gene amplification; we were unable to differentiate between alleles in heterozygous samples. This left analysis to be based on patterns of heterozygousity within the HVRs of the DLA-DRB1. Ideally, for future studies the alleles would need to be sub cloned for resolution of heterozygous samples.

Limitation with DLA Haplotyping

DDGE is an appropriate method in which to determine the allele present with the proper controls. This study was limited by the number of homozygous samples sequenced. Ideally there would be a homozygous control for each allele that could present in the GSD. This would also allow for the determination of new alleles present in the GSD.

Limitation in PCR / Argarose Gel Analysis of APOE, IL4R, HFE and TGFB1

PCR is one of the most common methods for gene identification. We developed primers based on the expressed DNA associated with different isoforms within the genes. Often these genes are thousands of base pairs long and the product we are examining is only hundreds of base pairs in length. While we directed analysis to the region in which the isoforms were present we may have missed a significant change elsewhere in the gene. Since we targeted an area which is variable by nature the primers were not specific to any one form. This lack of specificity allowed us only to note that there are different isoforms present based on the priming efficiency. In future studies sequence specific primers along with sequencing of PCR product can be used to verify the isoform present.

Limitation in PCR of SOD1

Primer selection for PCR of selected genes is often done based on conserved regions within in the DNA when making comparison to another species. Often these primers do not select for an extended length within the gene. Increasing the length of the PCR product to include more of the expressed gene would increase the chance of finding mutations within the gene.

Summary

DLA- DRB1 in MS and GSDM

German Shepherd degenerative myelopathy is a chronic, demyelinating neurodegenerative disease of the central nervous system. In order to investigate underlying genetics of this disease, we have hypothesized a genetic based immune-mediated etiology similar to MS in human beings. In this study, we demonstrated the presence of distinct patterns of heterozygousity in the DLA-DRB1 of GSDM samples. In human, the HLA-DRB1 is a genetic hallmark of MS. They have routinely demonstrated HLA-DRB1*15 in MS patients. In human, therefore, the HLA-DRB1 is not only used for aid in diagnosis but also for tracking of familial predisposition to MS. In the current study, the presence of a heterozygous pattern throughout the DLA-DRB1 is suggestive of an allelic associate like that of the HLA-DRB1. Although there was no resolution between heterozygous alleles, our results suggested the presence of two distinct alleles in GSDM

DLA- DRB1 in MS and GSD

Although no haplotype led to an association to GSDM, it supports the theory the DLA has minimal intrabreed variation (Kennedy et al. 2002). The findings in this studying support the existing data on the inheritance of the DLA within the GSD. The alleles determined were found to have similar frequencies within the GSD to the published data.

MBP in MS and GSDM

Findings of this study suggest MS and GSDM may share not only pathologic features, but also genetic factors involved in their pathogenesis. In this study we found disparity in product size is suggestive of a 70 bp tandem repeat (TGGA) deletion that correlates to the same deletion found in a population of MS patients in Finland, Pihlaja et al. A product of ~350bp was found in 88% of dogs with GSDM and only in 57% of healthy GSDs.

Genetic Associations in RRMS, PPMS and GSDM

In the current study, changes in PCR product of APOE, IL4R were seen; where there were no changes within the TGFB1 or HFE. In human studies of APOE in MS APOE-4 isoform was found more commonly in PPMS patients; IL4R isoform R551 was also found to be predominant in human patients presenting with more severe forms of MS like that of PPMS. of other neurological disorders, giving the test specificity of 91% for MS patients (Lunding, Midgard et al. 2000). Importantly, the presence of elevated IgG and unique CSF oligoclonal bands of IgG are similar in PPMS as we have described in GSDM patients.

In the current study, we demonstrated the presence of variations within the APOE and IL4R. The fact that variations were seen suggests different isoforms present in GSDM as compared to controls. Although it will be important to resolve the isoforms present in future studies.

SOD1 in ALS and GSDM

In the current study of SOD1, there were no changes noted based on the region analysed. In human studies changes in SOD1 are hallmark for ALS. Although GSDM and ALS are both progressive neurological disorders of the CNS there are differences in the presentation of the disease. ALS does not present with sensory deficits. It also has not been shown to have elevated proteins nor oligoclonal IgG bands, both of which have been found to be present in MS and

GSDM. ALS either damages cranial nerve and spinal cord lower motor neurons or cortical upper motor neurons which project to the spinal cord leading to cortical atrophy. This has not been seen to be true for GSDM. In studying the distribution of lesions, O'Brien suggested that Corgis Myelopathy is distinct from GSDM. DM in smaller breeds of dogs such as the Pembroke Welsh Corgi may be more representative of ALS and changes in the SOD1 have been noted within the breed by O'Brien et al. Recent studies looking into microsatellite markers have shown some GSDs presenting with the same change in the SOD1 (Awano et al. 2009). SOD1 will still be an important gene in the study of neurodegenerative disease.

Conclusion

German shepherd dogs presenting with clinically diagnosed DM do have a distinct allelic association in the DLA region of the MHCII analogous to MS patients. In addition, there are genetic variations present in markers associated with PPMS. These facts support the genetic basis of GSDM and indicate that GSDM is genetically comparable to PPMS. The age of onset, lack of sex bias, time course, location of neurologic damage, pathology, and the genetic variations demonstrated in this study support GSDM as genetically analogous to PPMS. Further study will be needed to understand the significance of these findings and how they related to overall genetic predisposition to GSDM. This study has helped to advance GSDM as animal model of PPMS in human beings.

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BIOGRAPHICAL SKETCH

Jennifer Anne Cheeseman was born in Kissimmee, Florida. She received her Bachelor of Science degree in microbiology and cell science from the University of Florida, in December 2003. She then worked in small animal research at the University of Florida for five years where she did research on German Shepherd degenerative myelopathy. From July 2006 to present, she has been a master's student in veterinary medical science at University of Florida and also worked as a biological scientist under the guidance of Dr. Roger M. Clemmons. Currently, she has accepted a position at Emory University as a senior research specialist working on immune mediated responses in bone marrow and organ transplantation.