Identification of Risk Loci for Necrotizing Meningoencephalitis in Pug Dogs

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Abstract

Due to their unique population structure, purebred dogs have emerged as a key model for the study of complex genetic disorders. To evaluate the utility of a newly available high-density canine whole-genome array with 170,000 single nucleotide polymorphisms (SNPs), genome-wide association was performed on a small number of case and control dogs to determine disease susceptibility loci in canine necrotizing meningencephalitis (NME), a disorder with known non-Mendelian inheritance that shares clinical similarities with atypical variants of multiple sclerosis in humans. Genotyping of 30 NME-affected Pug dogs and 68 healthy control Pugs identified 2 loci associated with NME, including a region within dog leukocyte antigen class II on chromosome 12 that remained significant after Bonferroni correction. Our results support the utility of this high-density SNP array, confirm that dogs are a powerful model for mapping complex genetic disorders and provide important preliminary data to support in depth genetic analysis of NME in numerous affected breeds.

Key words: canine, genome-wide association, haplotype, major histocompatibility complex, necrotizing meningencephalitis, single nucleotide polymorphism

Necrotizing meningencephalitis (NME) is an idiopathic inflammatory disorder of the central nervous system (CNS) that primarily affects young to middle aged toy breed dogs (Cordy and Holliday 1989; Stalis et al. 1995; Higgins et al. 2008). Inflammation in NME is characterized by mixed mononuclear cell infiltrates within the cerebral hemispheres and cortical leptomeninges with common clinical signs including seizures, depression, behavior change, circling, and visual deficits (Cordy and Holliday 1989). Similar to severe non-prototypical forms of multiple sclerosis (MS) such as Marburg variant (Bradl and Lassmann 2009; Hu and Lucchinetti 2009), NME is overrepresented in females, is rapidly progressive, and often carries a grave prognosis despite aggressive immunosuppressive treatment (Greer et al. 2009, 2010). NME initially was identified in Pug dogs in the late 1960s (Cordy and Holliday 1989) and is known to have a strong familial association in this breed (Cordy and Holliday 1989; Greer et al. 2009). Studies of Pugs with NME suggest that there are likely multiple genes that contribute to disease phenotype (Greer et al. 2009) and a recent genome-wide study of simple tandem repeat markers in this breed identified regions of disease susceptibility within dog leukocyte antigen (DLA) class II, similar to major histocompatibility complex (MHC) loci previously identified in MS and other proposed autoimmune diseases (Greer et al. 2010).

Purebred dog populations provide a unique opportunity for mapping genetic traits and recent technological developments have made it possible to leverage dogs as a model for the study of human genetic disease (Wilbe et al. 2010). The extensive linkage disequilibrium within breeds allows successful genome-wide mapping of traits and disease risk using smaller numbers of cases and controls than typically are required in a human-based study (Sutter et al. 2004; Lindblad-Toh et al. 2005), and dogs and humans share similar physiology with over half of the known canine diseases having a similar phenotype to analogous human diseases (Ostrander and Kruglyak 2000). Significant
advances in canine genomics, including publication of a high-quality draft genome sequence and identification of 2.5 million single nucleotide polymorphisms (SNPs), have facilitated mapping of simple and complex canine genetic traits (Lindblad-Toh et al. 2005; Karlsson et al. 2007; Wilbe et al. 2010), and genome-wide SNP arrays with coverage of up to 50,000 SNPs have been used successfully to map canine traits (Karlsson et al. 2007; Awano et al. 2009; Wilbe et al. 2010). The objective of this investigation was to employ a newly available high-density array to evaluate canine NME, a disorder with a presumed autoimmune etiology and complex mode of inheritance that has clinical similarities to atypical fulminant variants of MS. The identification of genetic risk factors should improve our understanding of NME pathophysiology, increase our ability to identify at risk and affected dogs, allow institution of targeted therapy, and ultimately may help in the identification of similar genetic factors that are associated with the development of rapidly progressive MS in people.

Materials and Methods

Study Population

Purebred Pug dogs were used for the case–control genome-wide association study. Cases were verified to have NME based on signalment, clinical history, and independent evaluation of hematoxylin and eosin brain sections by a veterinary neuropathologist. Cases ranged in age from 4 to 84 months (mean = 18 months, median = 26 months) and consisted of 11 males and 19 females. Control dogs had no evidence of neurological or autoimmune disease, ranged in age from 5 to 204 months (mean = 60 months, median = 48 months) at the time of sample collection, and consisted of 30 males and 38 females. Control dogs were followed for 18 months after sample collection to verify that they did not develop neurological or autoimmune disease.

SNP Genotyping

Genomic DNA was isolated using the Qiagen (Valencia, CA) Gentra Puregene Tissue Kit or Qiagen DNeasy Blood and Tissue Kit. SNP genotyping was performed with the Illumina (San Diego, CA) CanineHD Genotyping BeadChip using the Illumina BeadArray reader following the manufacturer’s protocol (Stein et al. 2010).

Statistical Analysis

Genotyping was performed on 98 dogs, including 30 NME cases and 68 controls. Genome-wide analysis was performed.
with PLINK (Purcell et al. 2007). Concordance on duplicate samples was 99.96%. Only samples with a call rate of >95% were included, resulting in analysis of 28 NME cases and 66 controls. A total of 172,115 SNPs were genotyped. Classic multidimensional scaling (Purcell et al. 2007) using a call rate of >97% and minor allele frequency (MAF) of >0.10 was performed on 85,366 SNPs to determine population stratification, and 21 controls that were not clustered with the main population of dogs were excluded resulting in a final population of 28 NME cases and 45 controls for analysis. These 45 control dogs ranged in age from 5 to 204 months (mean = 80 months, median = 48 months). Prior to analysis, 7,324 SNPs were excluded for failure to reach the call rate threshold (>95%), and 81,001 SNPs were excluded for failure to reach the MAF threshold (>0.05). In total, 86,692 SNPs were used for analysis. Bonferroni correction was applied to account for multiple hypothesis testing with a resulting $P$ value of $5.77 \times 10^{-7}$ across 86,692 SNPs for genome-wide significance. To further evaluate genome-wide significance, MaxT permutation testing (Purcell et al. 2007) of 100,000 permutations was applied.

### Results

Initial genotyping was performed on 30 NME cases and 68 controls across 172,115 SNPs. After quality filtering and exclusion of population outliers (Supplementary Material, Supplementary Figure S1), analysis of 28 NME cases and 45 controls across 86,692 SNPs identified 2 disease-associated loci that reached genome-wide significance with correction for multiple hypothesis testing. The strongest association was on chromosome 12 where 35 SNPs within the DLA class II region reached genome-wide significance after Bonferroni correction (raw $P$ value for Bonferroni genome-wide significance $< 5.77 \times 10^{-7}$) with the highest SNP having an odds ratio of 16.1 (95% confidence interval [CI]: 4.7–55.5) (Figure 1a and Table 1). Permutation testing using
100 000 permutations identified an additional 4 SNPs that reached genome-wide permuted significance within the DLA II locus and a second region of significance within the STYX gene on chromosome 8 ($P_{raw} = 2.11 \times 10^{-6}$, $P_{permuted} = 0.045$) with an odds ratio of 5.9 (95% CI: 2.7–12.5) (Figure 1b and Supplementary Material, Supplementary Table S1). To account for the fact that several of the control dogs were younger than the mean age of disease onset at the time of sample acquisition, the data were reanalyzed excluding all control dogs less than 24 months of age. Both the DLA and chromosome 8 regions remained significant with Bonferroni correction and permutation testing, respectively, but the significance was not improved by this exclusion (data not shown).

Haplotype analysis using Haploview (Barrett et al. 2005) identified 19 haplotype blocks across a 4.1 Mb region of DLA II on chromosome 12, all of which were associated with an increased risk for developing NME with $P$ values ranging from $2.1 \times 10^{-3}$ to $1.13 \times 10^{-8}$ (Figure 2 and Supplementary Material, Supplementary Table S2). Manually forcing all of these haplotype blocks into a single haplotype resulted in the creation of a 4.1 Mb haplotype containing 241 SNPs. This haplotype was common and strongly associated with an increased risk of developing NME (case frequency 85.1%, control frequency 38.4%, $P = 7.97 \times 10^{-5}$). Haplotype analysis of the STYX region of chromosome 8 identified 4 haplotypes (Figure 3). The most significantly associated and common haplotype spanned the STYX and GNPNA11 genes and was protective based on phenotype ($P = 1.43 \times 10^{-5}$) (Supplementary Material, Supplementary Table S3). This block also contained 2 additional haplotypes significantly associated with NME risk ($P \sim 0.005$, data not shown).

**Discussion**

Genome-wide analysis of NME in Pug dogs identified 2 disease-associated loci, including a strong association with DLA II. Although recognition of self-antigen has not been demonstrated definitively as a mechanism of pathogenesis in NME, CNS anti-astrocytic antibodies have been identified (Toda et al. 2007), and the strong DLA II association further supports an autoimmune etiology. Similar to our findings in NME, most autoimmune diseases are polygenic with MHC II polymorphisms having the strongest disease association (Lincoln et al. 2005). Haplotype analysis of the DLA II region identified a large common block strongly associated with altered disease risk. The large number of genes present within this haplotype precludes the precise identification of the associated gene without additional fine mapping and sequencing of this region. Although initially described in the Pug, NME has now been described in numerous other breeds including the Maltese (Stalis et al. 1995) and Chihuahua (Higgins et al. 2008) with identical clinical presentation and pathology suggesting a similar etiopathogenesis among these breeds (Stalis et al. 1995; Higgins et al. 2008). Fine mapping across breeds should allow identification of smaller disease-associated haplotypes in this region (Karlsson et al. 2007; Parker et al. 2007).
The role of STYX and GNPNAT1 in NME also require further characterization. STYX, serine/threonine/tyrosine interacting protein, is a pseudophosphatase that lacks intrinsic catalytic activity and is structurally similar to members of the dual-specificity phosphatase subfamily of protein tyrosine phosphatases (Wishart et al. 1995). The only documented role of STYX is in normal sperm formation in mice (Wishart and Dixon 2002). The STYX protein has been found in numerous tissues in mice including brain (Wishart et al. 1995), but its presence and role in immune cells have not been determined. Protein tyrosine phosphatases play a key role in immune system function including lymphocyte...
activation, with mutations in \textit{PTPN22} having been documented in association with autoimmunity (Vang et al. 2005). Less is known about the role of pseudophosphatases in immune and inflammatory responses, although a mutation in the pseudophosphatase MTMR13 has been implicated in a form of Charcot–Marie–Tooth neuropathy (Laporte et al. 2003), documenting a role for these proteins in development and maintenance of nervous tissue. STYX also is known to bind to the calcineurin substrate CRHSP-24 (Wishart and Dixon 2002). Although calcineurin plays an important documented role in T cell activation, the role of CRHSP-24 is less clear. CRHSP-24 has been found ubiquitously in rat tissue, and its dephosphorylation is prevented by administration of the immunomodulatory drugs cyclosporine and FK506 (Groblewski et al. 1998). Interestingly, CRHSP-24 has been found ubiquitously in rat tissue, and its dephosphorylation is prevented by administration of the immunomodulatory drugs cyclosporine and FK506 (Groblewski et al. 1998). Interestingly, CRHSP-24 has been found ubiquitously in rat tissue, and its dephosphorylation is prevented by administration of the immunomodulatory drugs cyclosporine and FK506 (Groblewski et al. 1998).

\textbf{Supplementary Material}

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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