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Topical Review

Current Status of Genetic Studies of Exocrine Pancreatic Insufficiency in Dogs

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A B S T R A C T

Exocrine pancreatic insufficiency (EPI) is a disorder wherein the pancreas fails to secrete adequate amounts of digestive enzymes. In dogs, EPI is usually the consequence of an autoimmune disease known as pancreatic acinar atrophy. Originally believed to be a simple autosomal recessive disorder, a test-breeding recently revealed that EPI has a more complex mode of inheritance. The contributions of multiple genes, combined with environmental factors, may explain observed variability in clinical presentation and progression of this disease. Research efforts aim to identify genetic variations underlying EPI to assist breeders in their efforts to eliminate this disease from their breed and provide clinicians with new targets for therapeutic intervention and/or disease prevention. Genome-wide linkage, global gene expression, and candidate gene analyses have failed to identify a major locus or genetic variations in German Shepherd Dogs with EPI. Recently, genome-wide association studies revealed numerous genomic regions associated with EPI. Current studies are focused on alleles of the canine major histocompatibility complex. In this article we review findings from scientific investigations into the inheritance and genetic cause(s) of EPI in the purebred dog.

Exocrine pancreatic insufficiency (EPI) is a condition caused by a failure of the pancreas to secrete sufficient quantities of digestive enzymes. EPI affects many breeds, but is most prevalent worldwide among German Shepherd Dogs (GSDs), in which it is an acquired condition that generally becomes apparent between 1 and 5 years of age.[1,2] Clinical screening for EPI is accomplished through measurement of serum canine trypsin-like immunoreactivity (cTLI). Very low values (< 2.5 μg/L) are diagnostic for EPI, whereas values between 5 and 35 μg/L are considered normal.[3] Although there are several underlying causes for EPI, in dogs it most often results from selective loss of the digestive enzyme-producing acinar cells of the pancreas.[4] This disorder is termed pancreatic acinar atrophy (PAA) and is definitively diagnosed through histologic examination of the pancreas. PAA is likely an autoimmune disorder because lymphocytic infiltration of the pancreatic acinar tissue occurs concurrently with active tissue destruction.[5] In this article, the term EPI will be used when the underlying etiology is suspected or assumed to be PAA but is not histologically proven.

EPI is a disease for which a genetic test would be particularly desirable. Genetic testing is becoming commonplace in companion animal medicine, with more than 80 DNA tests currently available for mutations associated with diseases and morphologic characteristics of the purebred dog.[6] Genetic tests allow breeders to make educated choices to reduce the incidence of diseases or undesirable traits through the early identification of diseased dogs and carriers of recessive mutations. When genetic tests are unavailable, breeders often feel pressure to remove the parents and siblings of affected dogs from their breeding program, thereby reducing genetic diversity.

The results of serum cTLI assay are diagnostic for EPI, but mildly to moderately subnormal concentrations (2.5-5.0 μg/L) are not completely predictive of the presence of PAA, because it is possible for clinically and pathologically normal dogs to have one or more unusually low serum cTLI values, followed by normal test results.[2] Although 93% of affected dogs will show signs of maldigestion by the age of 4,[7] there is variability in age of onset and a very small proportion of affected dogs remain apparently asymptomatic throughout their lives. Normal histologic appearance of pancreatic biopsies obtained early in life do not guarantee that the animal will remain normal.[2] The goal of this article is to update the veterinary community regarding current knowledge of the hereditary basis for EPI and recent progress toward a genetic test.

Inheritance

Although nearly 70% of hereditary diseases of dogs are recessive,[8] many others are influenced by multiple genes and/or environmental factors. An increased incidence of EPI among certain breeds and within breeding lines is strongly suggestive of a heritable component.[1,9,10] Autoimmune diseases are notoriously complex because they may be influenced by genetic susceptibility and protective factors, and environmental triggers, such as drugs and viral infection.[11] Therefore, it is not surprising that investigations into the inheritance of EPI have been complicated by multiple underlying causes (e.g., PAA, chronic pancreatitis) and variability in both age of onset and clinical presentation.

Numerous investigations of EPI in the GSD,[2,9,10,12] Rough Collie,[13] and Eurasian[14] breeds have been carried out in the last 4 decades (Table 1). Early studies of GSD pedigrees from Germany and Finland led authors to propose an autosomal recessive mode of inheritance.[9,12] The production of affected progeny from unaffected parents, high inbreeding levels, and the identification of ancestors common to EPI dogs all supported a recessive inheritance pattern in families used in these studies.[9,12]

In 2002, Moeller and colleagues[10] identified 2 GSD families from the United States with a high prevalence of EPI. To investigate transmission, the authors used complex segregation analysis (CSA), a statistical approach wherein different inheritance models are applied to evaluate which one(s) best fit the observed pattern in a large family. CSA data indicated that a major gene, likely with a recessive pattern of

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Table 1
Inheritance Studies of EPI in Purebred Dogs

Breed	n_{total}	n_{EPI}	Approach	Conclusions	Reference
GSD	52	19	Pedigree analysis	Autosomal recessive	12
GSD	59	14	Pedigree analysis	Autosomal recessive	9
Collie	51	51	Pedigree analysis	Autosomal recessive	13
GSD	135	19	Segregation analysis	Autosomal recessive	10
Eurasian	58	16	Segregation analysis	Autosomal recessive	14
GSD	8	4	Test-mating	Polygenic	2

inheritance, is responsible for EPI in these families. An interesting finding from this study is that about half of the affected members from one family repeatedly had serum cTLI values diagnostic for EPI (< 2.5 μg/L), but never exhibited clinical signs of the disease. Histopathology in 2 asymptomatic family members, 8 and 12 years of age, confirmed severe, diffuse acinar atrophy. All affected dogs in the second family showed clinical signs.[10]

In 2010, Westermarck and colleagues[2] published findings from a test mating of 2 unrelated GSDs with symptomatic EPI. Out of a litter of 6 surviving puppies, 4 had no clinical or pathological signs of EPI. Measurement of serum cTLI was completed numerous times throughout the dogs' lives; each dog had at least 1 subnormal serum cTLI value (2.5-5.0 μg/L). Each of the 4 dogs died of natural causes between 8 and 13 years of age, and all pancreata were grossly and histologically normal. One of the 6 puppies developed clinical EPI (cTLI = 0.3 μg/L) at 25 months of age, whereas another showed low serum cTLI values (2.1 μg/L) and partial pancreatic atrophy at 5.5 years of age. Although the latter dog was asymptomatic, it continued to have subnormal or diagnostic serum cTLI values. When the dog died at 12 years of age, the pancreas was abnormal, with some areas similar to end-stage PAA. It is not known whether a year of treatment with immunosuppressive drugs changed the progression of EPI in this dog.[2] If EPI were a simple autosomal recessive disorder, all progeny from this test mating would have developed the disease. This study provides the first conclusive data that EPI is a genetically complex disorder.

Linkage and Association Analyses

Classical linkage analysis is a pedigree-based method for the identification of chromosomal regions "linked" with a particular disease. When a de novo mutation occurs, neighboring genetic variations will be coinherited with it. Linkage analysis requires samples of genetic material from multigenerational families having both affected and unaffected members, and a genome-wide set of polymorphic markers. The most commonly used markers are termed "microsatellites" and are short repeating DNA sequences. Microsatellites are particularly informative because they have multiple alleles, making it possible to trace the inheritance of chromosomes through a pedigree. Once genotypes are determined for each pedigree member, a logarithm of the odds (LOD) score is calculated to determine if the microsatellite marker is linked with the phenotype. By convention, a minimum LOD of 3 is considered evidence for linkage, but this threshold is more accurately determined by the population size and structure and may vary from study to study.

Genomic DNA collected from members of the 2 families analyzed in the 2002 study by Moeller and colleagues[10] were used to carry out linkage studies for EPI.[15] The GSD kindred were genotyped for 384 microsatellite markers spanning all 40 canine chromosomes. No LOD scores reached significance (≥ 3). Additional family members and markers were used to further evaluate a region on chromosome 3, which contained 2 markers having the highest results. Unfortunately, the maximum LOD score obtained in this study was 2.5, and no genomic regions linked with EPI were detected.[15]

In 2005, the canine reference sequence ("genome") was published, providing new resources for the identification of genetic variations that contribute to complex traits.[16] In addition to generating dense coverage of the 2.4-Gb genome from a female Boxer, researchers also assembled partial sequences for 10 other diverse breeds.[16,17] These sequences were compared in order to identify single nucleotide polymorphisms (SNPs), which are sequence variations that occur at a single base pair. Although most SNPs only have 2 alleles (e.g., C or T), they are useful as genetic markers because they are highly conserved and abundant, and found throughout the genome. Moreover, SNPs can be rapidly genotyped with high-throughput, microarray-based technologies. More than 2 million SNPs have been identified in the dog, and informative subsets have been selected for arrays that can simultaneously genotype up to 170,000 SNPs.[18]

SNP arrays provide comprehensive genetic profiles of individuals, which are necessary for genome-wide association (GWA) approaches. GWA studies are based on the same principles as classical linkage analysis, but use unrelated populations rather than families. Individuals are divided into 2 categories, usually cases and controls, and allele frequencies are compared between the 2 groups. A SNP allele that is significantly more prevalent in one group is said to be "associated" with the phenotype, and provides researchers with a chromosomal locus for further investigation. GWA approaches are well-suited for the study of complex diseases because they can uncover susceptibility and protective loci and variants having even minor effects on the phenotype. In the dog, GWA studies have revealed loci associated with several complex diseases, including intervertebral disk calcification,[19] mitral valve disease,[20] early-onset retinal degeneration,[21] and dilated cardiomyopathy.[22]

Data from the first GWA study for EPI were published in 2012.[23] A cohort of 100 EPI-affected and 79 control GSDs was assembled primarily from the United States, but also from Canada, The Netherlands, and Germany. Genetic profiles were generated for each dog using 48,415 SNPs of proven analytical reliability. Analyses indicate no major loci associated with EPI, but rather multiple regions of association (Fig 1). Only 2 regions, on chromosomes 7 and 12, were supported by multiple SNPs.[23] These data suggest that EPI in the GSD may be governed by multiple loci with small effects, or that it may have several different genetic causes.

Global Gene Expression Analysis

Measurement of global gene expression is another approach for the identification of candidate genes and/or pathways involved in EPI. Transcript levels of thousands of genes can be simultaneously determined with microarrays. Briefly, RNA is extracted from the tissue of interest, usually the tissue most affected by the disease (e.g., the pancreas in EPI), and is hybridized to the microarray chip. Relative amounts of RNA in the tissue will be detected on the chip, and transcript levels in affected and normal dogs are compared. Genes with increased or decreased expression in affected animals may be involved in the disease process. Dramatic changes in individual genes may indicate strong candidate genes; smaller but significant changes in groups of genes may point to important biochemical pathways. Microarrays can thus reveal important clues in disease cause and pathogenesis.

In 2005, Clark and colleagues[15] probed a microarray containing 22,748 test sequences using RNA from the pancreata of 2 GSDs with

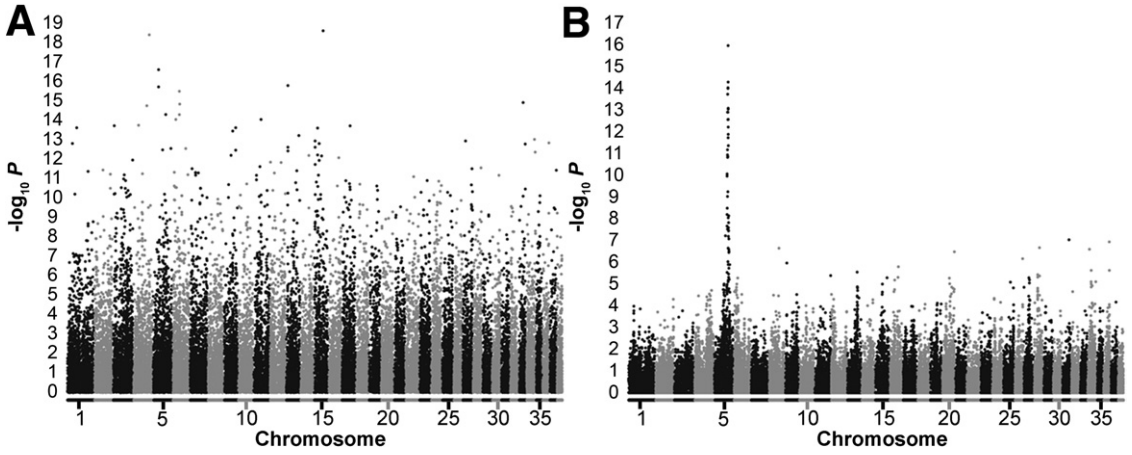

Fig. 1. Manhattan plots showing the results for GWA analyses using 179 GSDs. *P* values (-$\log_{10}$ *P*) (y-axis) for each SNP are plotted against chromosomal position (x-axis) for the 38 canine autosomes. (A) One hundred EPI-affected GSDs versus 79 healthy GSDs show no major signals. (B) For comparison, the same population was used to map white fur color, a simple recessive trait. Seven white GSDs versus 172 nonwhite GSDs show a strong association with the position of the causative gene on chromosome 5.

histologically confirmed PAA, 1 healthy GSD, and 2 healthy mixed-breed dogs. The study identified 244 differentially regulated genes: 231 are upregulated and 13 are downregulated by 2-fold or more in PAA-affected GSDs. The results showed differential regulation of a functionally diverse population of genes. Of particular interest were genes involved in immune function. Transcript levels for one such gene, *DLA-88*, are increased 4.6-fold in affected dogs.[15]

Expression studies may have been confounded by the nature of the disease and the use of end-stage tissues. Many relevant gene signatures may no longer be present in affected tissues because of total atrophy of the acinar cells. Other gene signatures may represent the pathological process currently taking place in the tissue, rather than the cause of that process.

Candidate Gene Analysis

Candidate gene analysis involves targeting specific genes and sequencing them for mutations that could be causative for the observed phenotype. Genes of interest may be identified through techniques like those described above, based on disease similarity between breeds or species, or through biochemical processes that are hypothesized to be involved in disease pathogenesis.

An intriguing candidate gene, *Serpini2*, was identified in a mouse model of pancreatic insufficiency.[24] In these mice, a deletion in *Serpini2* results in acinar cell apoptosis. The mutation is inherited in a simple autosomal recessive pattern, and as in dogs with EPI, acinar cell destruction is progressive but not congenital, and symptoms are alleviated by pancreatic enzyme supplementation. Sequencing of the 8 exons and surrounding genomic boundaries of *Serpini2* in GSDs with EPI failed to identify any genetic variations to implicate *Serpini2* in canine EPI (Clark, unpublished).

The cholecystokinin and cholecystokinin A receptor (CCKAR) genes were investigated in Eurasian dogs having EPI.[14] The genes were selected through a combination of function (involvement in pancreatic enzyme secretion) and position (CCKAR is located on chromosome 3, within a candidate region identified in linkage studies).[15] Linkage analyses in Eurasian dogs failed to find linkage between markers for these genes and EPI.[14] Sequencing of CCKAR DNA in the GSD also failed to reveal any causative mutations (Clark, unpublished).

Gene expression studies identified another gene of interest, *glycoprotein 25L* (*gp25L*), which encodes an endoplasmic reticulum protein.[15] Transcript levels of this gene, located within a region of interest on chromosome 3, are more than 500-fold reduced in the pancreata of affected GSDs. However, sequencing of the coding region and splice sites of *gp25L* failed to reveal any causative mutations.[15]

Conclusion

EPI is a disease complex in presentation and inheritance. Families used for inheritance and linkage studies had great phenotypic variability, having both clinically affected and asymptomatic dogs in the same litter.[15] Although data from numerous independent studies were compatible with an autosomal recessive mode of inheritance, a test-mating unequivocally excludes this possibility, suggesting instead a polygenic mode.[2] GWA analysis in a large population of unrelated GSDs did not identify a major locus, but rather revealed numerous associated chromosomal regions.[23] Taken together, these data clearly point to the involvement of multiple genetic and/or environmental factors in EPI. It remains unclear whether there are different genetic causes in different populations, multiple genetic variants with additive effects, or both genetic and environmental factors.

Current genetic studies are focused on chromosome 12. This region was prioritized because several SNPs associated with EPI were proximal to the dog leukocyte antigen (DLA), which is part of the major histocompatibility complex.[23] The DLA is comprised of several genes important for immune recognition and regulation, and multiple associations with alleles of these genes have been identified for other canine autoimmune diseases.[25-27] The overexpression of *DLA-88* provides further evidence for the involvement of the DLA.[15] These ongoing investigations, which are focused on 4 polymorphic DLA genes, may reveal susceptibility and/or protective alleles for EPI.

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