A non-synonymous change in adhesion G protein-coupled receptor L3 associated with risk for Equine Degenerative Myeloencephalopathy in the Caspian Horse.

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Abstract:

Equine Degenerative Myeloencephalopathy (EDM), a neurological disease of young horses, causes progressive development of symmetric ataxia predominantly in the pelvic limbs. EDM is likely inherited and with no known treatment affected horses frequently need euthanasia. Alpha-tocopherol deficiency during early life appears to contribute to the phenotype. This study sought to identify any genetic variants correlated with EDM in Caspian foals. Two half-sibling EDM diagnosed cases were genotyped at 52,063 loci and evaluated by the Autozygosity by Difference statistic. Additional horses not affected by EDM were used for genetic comparison to identify regions unique to the case phenotype. The associated region on chromosome 3 contains only one gene encoding adhesion G protein-coupled receptor L3 (*ADGRL3*). *ADGRL3* is a member of the latrophilin subfamily of G-protein coupled receptors and may contribute to Attention Deficit/Hyperactivity Disorder in humans and hyperactive motor function in mice and zebrafish. Analysis of the predicted coding regions for Equine *ADGRL3* in affected horses revealed a non-synonymous SNP at Chr3:71,917,591 bp. Caspian and Caspian cross relatives (n=81) of the two initial cases, as well as unrelated horses from similar breeds (n=130, including Arabians, American Miniatures, and Shetlands) possessed this allele at 5% frequency, with no homozygotes observed within the non-Caspian breeds. This study suggests that a polymorphism in *ADGRL3* could contribute to a genetic predisposition to Caspian Horse EDM.

Keywords: Latrophilin-3; autozygosity by difference; sequencing; equine neuroaxonal dystrophy

1. Introduction

Equine Degenerative Myeloencephalopathy (EDM), a more severe variant of equine neuroaxonal dystrophy (eNAD), is a progressive neurological disease found in young horses. Clinical signs generally develop within the first year of life and most commonly present as symmetric ataxia and weakness, affecting either the hind limbs only or all four limbs [1, 2]. Diagnosis is confirmed at necropsy by observation of axonal degeneration in specific neurons within the caudal medulla oblongata and cervicothoracic spinal cord [1]. EDM has been previously reported in Arabians, Appaloosas, Lusitanos, and Welsh Ponies, among various other breeds, including mixed breeds [3-7]. Evidence for a genetic influence for the disease is provided by breeding trials and risk factor analysis, however different inheritance patterns have been observed across different breeds [5, 7-11].

Vitamin E, specifically alpha-tocopherol, deficiency is associated with the incidence and severity of EDM [12]. Alpha-tocopherol supplementation reduces the severity and incidence in genetically predisposed foals [8, 11]. However, all reported cases may not be deficient in alpha-tocopherol at the time of diagnosis [9, 11]. It was recently demonstrated that the risk period where alpha-tocopherol deficiency may affect the phenotype is in the first 6 months of life [13].

The Caspian breed, originally developed in Northern Iran, is a rare breed that comprises approximately 1,600 horses worldwide. Close to extinction in the middle 20th century, an American horse breeder rediscovered the Caspian horse and promoted the introduction of this small breed into many countries. Caspian horses have undergone several population bottlenecks and founding events, resulting in decreased heterozygosity [14, 15]. This lack of heterozygosity increases the chance that deleterious recessive alleles will be observed in the

current population and provides a unique opportunity for genetic mapping efforts. Without monitoring for possible genetic faults, the future of the Caspian is uncertain, especially if a deleterious or sub-lethal recessive trait is carried within the population.

Two EDM cases submitted for genetic evaluation were genotyped on the equine 54K SNP chip and analyzed by a novel method, Autozygosity by Difference (ABD), to identify possible regions contributing to the disease. Through fine-mapping, a candidate variant was found in association with the disease and may be contributing to genetic predisposition, in conjunction with environmental factors, for development of EDM within the Caspian breed.

2. Materials & Methods

2.1 Proband Cases

Two half-sib yearling foals, a Caspian and Caspian cross, sharing a sire, were examined because of progressive and symmetrical ataxia of several months duration. They were scored using a modified grading scale for ataxia and neurologic deficits from 0-5, with 0 being normal and 5 being recumbent [16]. Both foals scored a 4 in the pelvic limbs and 1-2 in the thoracic limbs. The foals were from the same farm and it was reported that some previous weanling and yearlings from this farm had been euthanized because of similar clinical signs. Necropsy found bilateral symmetric demyelination and axonal loss around the spinocerebellar tracts in both cases, confirming the diagnosis as EDM. One case had low serum alpha-tocopherol concentration of 161 ug/dL (normal range 200-400 ug/dL). Testing was performed after the yearling had access to some pasture. It was assumed the other case was deficient because of a history of poor quality hay, limited pasture, and no additional alpha-tocopherol supplementation in the herd.

2.2 Horses

All horses had hair or tissue samples taken following protocols approved by the Cornell University Institutional Animal Care and Use Committee. All horse owners signed a consent form prior to their horses being sampled. Eighty-one Caspian and Caspian-cross relatives, as well as one Shetland (dam of a case) were submitted voluntarily by owners. The Caspian horses available shared common ancestry within 3-4 generations and we accounted for this using family-based statistical tests. An additional unrelated 130 horses were utilized to establish the allele frequency (13 Shetlands, 48 Arabians, and 69 Miniatures). These additional horses were selected to ensure each one was not related to another within one generation.

2.3 DNA extraction

Genomic DNA from the affected foals was obtained from tissue samples using a Qiagen Puregene Kit following the manufacturer's recommended protocol. Genomic DNA from all other horses was extracted from hair bulbs using a modified Qiagen Puregene Protocol as previously published [17].

2.4 Autozygosity by Difference method

Autozygosity by Difference (ABD) identifies homozygous regions of the genome using genome-wide SNP data [18]. The two cases and 90 controls from other studies [19, 20] were genotyped on the Equine 54K SNP Chip. SNPs located on the sex chromosomes were excluded leaving 52,063 SNPs for analysis. ABD identifies the most common homozygous genotype (CHG) in the cases at each individual SNP. A run of homozygosity (ROH) is considered to be two or more adjacent CHG SNPs. Each ROH is scored on all chromosomes for each case and control. Each SNP in a ROH obtains a score based on the number of SNPs

in that ROH. Each SNP is analyzed by comparing the means for cases and controls. The mutation should be in the region with the highest score difference between cases and controls. Significance of the identified scores is calculated by permutation. This is achieved by repeatedly rescoring the dataset with phenotypes randomly allocated to subjects and including the original cases and controls in the permutations 1,000 times (N). The probability of each score is the proportion of occasions when that score, and all those greater than it, was achieved, out of N times the number of SNP tested.

2.5 Adhesion G Protein–Coupled Receptor L3 Sequencing

Adhesion G Protein–Coupled Receptor L3 Sequencing (*ADGRL3*) exons were inferred based on homology with the human, mouse, rat, and bovine genes using the UCSC Genome Browser. Primers covering the orthologous exons, 5' end, and 3' end were designed based on the equCab2.0 assembly from the UCSC Genome Browser using Primer3 software [21] and purchased through Integrated DNA Technologies (Coralville, Iowa) (Supplemental Table 1).

Polymerase chain reaction (PCR) products from all primer sets from both cases and a control were sent to Cornell University Life Science Core Laboratory Center for Sequencing using Applied Biosystems Automated 3730xl DNA Analyzers and run according to manufacturer's recommendations.

Sequences were compared to detect variation using CodonCode Aligner (CodonCode Corp, Dedham, MA). These were also compared to the reference genome utilizing the UCSC Genome Browser [22]. DNA changes that resulted in amino acid changes were tested for a predicted functional effect using PROVEAN [23].

2.6 Statistical Analysis of Association

Genotypic and allelic frequencies were used to determine statistical significance through association using the recessive model for the entire dataset (n=212) and a family based TDT for the Caspian individuals (n=81) using PLINK 1.9 [24]. Hardy-Weinberg Equilibrium was tested within breed using an exact test in R [25, 26].

3. Results

3.1 Autozygosity by Difference

Through the ABD method, a run of homozygosity was found on Chromosome 3 from 71,381,589-73,566,775 bp (Figure 1). This region has a difference score of 376.47, which is significantly higher ($p<0.001$) than the rest of the genome with an average score of 7.11. Only one gene is located within this region, *adhesion G protein-coupled receptor L3 (ADGRL3)*. The ABD score difference pattern suggests an autosomal recessive pattern of inheritance.

3.2 *ADGRL3* Sequencing

Four SNPs were identified via Sanger Sequencing; two in exons, one in an intron, and one in the 3' UTR (Supplemental Table 2). The SNP at chr3:71,770,084 was prioritized for further evaluation because it is a non-synonymous change and followed the recessive inheritance pattern indicated by the ABD mapping results. This SNP causes an amino acid change from asparagine to serine and resulted in a PROVEAN score of -0.962, indicating a slightly deleterious mutation. Two predicted *ADGRL3* coding sequences, an affected and an unaffected, were submitted to GenBank under the following accession numbers: KJ526819 and KJ526820.

3.3 Allele Frequency in source herd & other breeds

Genotypic and allelic frequencies are seen in Table 1. There were no homozygotes found in the other breeds tested. Association with the disease using a family based TDT for the Caspian horse cohort (n=81) was also significant (p=0.0455). Testing for an allelic association using the recessive model (n=212) revealed a significant association ($p<0.001$). We failed to reject the null hypothesis of Hardy-Weinberg equilibrium for this non-synonymous SNP within each breed.

4. Discussion

ADGRL3, also known as *LPHN3*, is a member of the latrophilin subfamily of secretin G protein coupled receptors that is expressed in the striatum, a part of the brain important for motor control and reward [27]. ADGRL3 is known to play a role in glutamatergic synapse development [28]. Loss of function in a homolog, lphn3.1, leads to a hyperactive motor phenotype in developing zebrafish and in the *ADGRL1* knock-out mouse [29, 30]. This study is the first to associate *ADGRL3* polymorphism with equine disease.

The only two living individuals possessing the homozygous EDM associated genotype have not yet exhibited signs of EDM. The two proband cases had limited access to green forage and were not supplemented with alpha-tocopherol, resulting in low serum concentrations. We know that supplementation of Vitamin E began on the farm after the initial diagnosis of EDM and hypothesize that these two living homozygous individuals may have had adequate levels of vitamin E during the early life risk period preventing them from displaying clinical signs or delaying the onset of EDM [13]. Further study and a larger sample size are needed to confirm the suspected interaction between vitamin E levels and the risk genotype.

This study demonstrates the feasibility of identifying recent recessive mutations in very small sample sizes using Autozygosity by Difference . However, the recessive inheritance pattern seen here contrasts with other studies of EDM in other breeds [1, 3, 9, 10]. EDM may be due to multiple breed-specific mutations preventing researchers from identifying a single causal mutation across multiple studies and different breeds.

5. Conclusions

The non-synonymous SNP identified in this study appears to be a genetic risk factor, working in conjunction with environmental factors, in the development of the EDM in the Caspian breed. However, observing no other homozygotes among Arabians, Miniatures, and Shetlands suggests that the homozygote state could also be a risk factor within those breeds. To validate this hypothesis, individuals diagnosed with EDM from other breeds should be genotyped at this SNP. This would determine if this SNP may also be a risk factor for EDM across all breeds or only for Caspian EDM. Further study is needed to confirm the suspected interaction between

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Tables

Table 1. Genotype & Allele Frequencies of Individuals tested for chr3:71,770,084 SNP

		Genotype Count & Frequency			Allele Frequency	
Breed	Number	AA	AG	GG	A	G
Arabian	48	46 *(95.8%)*	2 *(4.2%)*	0 *(0%)*	0.979	0.021
Mini[1]	69	61 *(88.4%)*	8 *(11.6%)*	0 *(0%)*	0.942	0.058
Shetland	14	11 *(78.6%)*	3 *(21.4%)*	0 *(0%)*	0.893	0.107
Caspian[2]	79	59 *(74.7%)*	18 *(22.7%)*	2 *(2.6%)*	0.861	0.139

1. American Miniature Horse
2. Includes 2 Caspian sired crossbreds, proband cases were excluded from allele frequency calculation

Figure legends

Figure 1: A) Plot of ABD score differences between cases and controls, showing a region of significance on ECA3. Higher ABD scores indicate SNPs are located in a region of longer ROH. B) shows the ABD scores for all SNPs on ECA3 with the highest ABD scoring SNPs circled; blue line indicates controls, red indicates cases, and green indicates the ABD score difference. C) Genome browser view of the region with the highest ABD score difference (UCSC Genome Browser, Santa Cruz, CA). *ADGRL3* is the only candidate gene within the region.

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