Homozygosity mapping approach identifies a missense mutation in equine cyclophilin B (PPIB) associated with HERDA in the American Quarter Horse

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Abstract

Hereditary equine regional dermal asthenia (HERDA), a degenerative skin disease that affects the Quarter Horse breed, was localized to \textit{ECA1} by homozygosity mapping. Comparative genomics allowed the development of equine gene-specific markers which were used with a set of affected horses to detect a homozygous, identical-by-descent block spanning \(\sim 2.5\) Mb, suggesting a recent origin for the HERDA mutation. We report a mutation in cyclophilin B (\textit{PPIB}) as a novel, causal candidate gene for HERDA. A c.115G\textgreater A missense mutation in \textit{PPIB} alters a glycine residue that has been conserved across vertebrates. The mutation was homozygous in 64 affected horses and segregates concordant with inbreeding loops apparent in the genealogy of 11 affected horses. Screening of control Quarter Horses indicates a 3.5\% carrier frequency. The development of a test that can detect affected horses prior to development of clinical signs and carriers of HERDA will allow Quarter Horse breeders to eliminate this debilitating disease.

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Keywords: Horse; HERDA; Skin disease; Inherited disease; Heterozygote detection

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Hereditary equine regional dermal asthenia (HERDA) is a degenerative skin disease predominantly found in the American Quarter Horse breed. The most common phenotypes are seromas, hematomas, and ulcerations that develop primarily along the dorsal aspect (Fig. 1A) and progressively worsen in frequency and severity with age. Loose, sloughing skin (Fig. 1B) is characteristic of affected tissue and often represents an early indication of the condition [1]. Severe lesions begin to develop on average at 1.5 years of age (Fig. 1C) and can deteriorate quickly (Fig. 1D) due to an inability to treat the condition. Consequently, the majority of affected horses are euthanized. Previous analyses have supported an autosomal recessive mode of inheritance and a carrier frequency between 1.8 and 6.5\% has been estimated [2].

Foals affected with HERDA rarely show symptoms at birth and areas that develop lesions are focal and nonuniformly distributed over the body. Many cases are not identified until the horses have a saddle on their backs, and lesions are most commonly found along the dorsal aspect, coincident with where the saddle would rest. Accordingly, HERDA may be related to a deficiency in wound healing or to dysregulation of inflammation. Histological examination does not definitively diagnose the disease, although subtle signs of thinned and shortened collagen fibers in the deep dermis and thinner skin thickness in affected regions suggest a general disorganization in comparison to control biopsies [1,3,4]. In two studies collagen 1 and collagen 3 content were indistinguishable in affected and unaffected horses [1,3].

Published case reports describing HERDA (also known as hyperelastosis cutis) have compared the disease to Ehlers–Danlos syndrome (EDS) [5]. Although the phenotype of loose skin (Fig. 1B) in affected areas is consistent with EDS, the normal quality of skin in unaffected areas of the body and the disease’s progressive nature suggest a distinct genetic cause. Researchers have focused histologically on the disorganization of collagen seen in affected tissue, yet unaffected tissue taken from affected horses (either before they show signs of the...
disease or simply in areas which are unaffected) appears healthy and consistent with control samples [6]. This indicates that the natural growth and development of skin of an affected horse may not be deficient with regard to de novo collagen metabolism. Whether the protein product of the gene responsible for HERDA directly interacts with collagen or more broadly has an effect on posttrauma collagen deposition, part of the wound-healing process, remains unclear.

The term quarter horse dates back to the 17th century and derives its name from their use in short quarter-mile races in colonial America. While there is no known definitive genetic makeup of the original Quarter Horses, imports of English racing stocks and horses that were introduced into North America by early Spanish explorers factored heavily into the breed in the 17th and 18th centuries. As settlers migrated westward across North America, the Quarter Horse became known for its versatility, strength, and agility, particularly as these qualities relate to the horse’s ability to work on ranches and aid in managing cattle herds. An official registry of the American Quarter Horse was begun in 1941 and as of today the breed is the largest in the world, registering 165,057 new horses in 2005 (AQHA Annual Report, 2005). While the majority of horses are kept for recreational purposes, racing and other performance events which emphasize the Quarter Horse’s unique capabilities serve as additional factors that affect breeding decisions within the population.

Here we report the first whole-genome scan used to identify a novel disease gene in the horse. While genome scans have been used to map the locus for cream dilution [7], gray coat color [8,9], and appaloosa coat color [10] in horses, the identification of equine disease genes has exploited human and mouse disease models and relied on a candidate gene approach. The unusual structure of horse families (half sibships) and their relatively long generation times create a challenge for obtaining multigeneration families that segregate a disease, particularly one which does not develop on average until 1.5 years of age [1]. Horses which become affected are often no longer owned by the breeder and full siblings of an affected horse are often never produced. The low incidence of HERDA resulted in our dataset consisting primarily of affected horses and very few samples from first-degree relatives when the study began. Since a common ancestor could be identified on both sides of an affected animal’s pedigree [1], a homozygosity mapping approach was taken to map the locus.

A scan of the genome identified regions identical-by-descent in affected HERDA horses on the q arm of ECA1 (closest to

Fig. 1. Phenotype of HERDA-affected horses. (A) A 2-year-old HERDA-affected stallion with mild lesions concentrated along the dorsal aspect (white circle), including early indications of loose, sloughing skin (white arrow). (B) Extensible skin in affected tissue can be more easily separated from the underlying fasciae. (C) A large hematoma developed at approximately 1.5 years of age along the left dorsal side of this affected horse. (D) Additional ulcerations and degeneration of the skin as seen on the right dorsal side of the horse shown in C.
marker AHT58). Fine-structure mapping with single nucleotide polymorphisms (SNPs) developed from the ~10-Mb interval further refined the region to ~2.5 Mb. Based on the genome annotations of human, chimpanzee, mouse, dog, and cow, there are roughly 20 genes in the candidate region. Sequence analysis revealed a nonsynonymous mutation in exon 1 of equine PPIB. This SNP segregates perfectly with HERDA and can be adopted for identifying carriers and screening foals to determine future disease status. The region of linkage disequilibrium within the affected samples is large, therefore additional functional analyses will be necessary to verify a causative link between the PPIB mutation and HERDA.

**Results and discussion**

**Mapping HERDA**

Samples used in the genome scan consisted of 38 affected HERDA horses and 44 unrelated control horses. Of 98 autosomal microsatellite loci tested, 13 exhibited significant differences between expected heterozygosity values and an unambiguous decrease of observed heterozygosity in the HERDA horses compared to the general population (Table 1). Potentially associated loci were further evaluated for significant differences in allele frequencies using a \(\chi^2\) test comparing the two sampled populations. Only HMS15 and HMS7, two markers that map ~18 cM apart on ECA 1[11], gave highly significant \(p\) values (Table 1).

To confirm association to HERDA, 52 samples (Fig. 2A) from 11 affected horses and 41 relatives were genotyped at nine loci on ECA1. The average distance between markers was 16.8 cM[11] and the two markers (HMS15 and HMS7) which were used to initially detect the reduction in heterozygosity within the affected population were replaced by alternative anchored markers for this stage of analysis. A maximum LOD score of 7.43 was generated at marker AHT58 (Fig. 2B). With the exception of flanking markers within 20 cM of AHT58, no other marker on ECA1 generated a LOD score >0.0 (average ~0.7).

**Fine-structure mapping**

Initially, 10 microsatellites (Fig. 3B) that were previously mapped by linkage or radiation hybrid analysis to a region near the AHT58 marker[11,12] were used to further refine the interval. Of 68 HERDA horses tested, 64 showed homozygosity centered around the AHT58 marker (Fig. 3B). Eight samples were homozygous at all 10 microsatellite markers, a region that spans 32 cM[11], and presumably represent the ancestral haplotype upon which the mutation arose. Fifty-six affected samples contained a smaller block of homozygosity either proximal and/or distal to but always containing the AHT58 microsatellite. Four samples diagnosed as affected in our dataset clearly deviated from the HERDA haplotype. Since the number of HERDA cases seen by veterinarians began to increase in the late 1990s[1], multiple lay articles informing Quarter Horse breeders of the disease have been published. Lacking a definitive histological method for diagnosing the disease, local veterinarians that suspected a case of HERDA often submitted blood samples to UC Davis in hopes of aiding the ongoing research into the genetic basis of the disease. Although care was taken to effectively screen submitted cases, issues of phenocopy and premature diagnosis are suspected to have led to the incorrect inclusion of horses in our affected dataset. The owners of these four genetically dissimilar (specifically in relation to markers on the q arm of ECA1) cases were contacted to determine the current status of each horse. Personal communication with one owner revealed that no further lesions had developed and the horse had begun training for performance events, consistent with a misdiagnosis. A second horse was resampled and the atypical genotype across

**Table 1** Identification of markers exhibiting increased homozygosity

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chrom.</th>
<th>Expected heterozygosity</th>
<th>Control</th>
<th>HERDA</th>
<th>(\chi^2) test of independence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASB41</td>
<td>1</td>
<td>0.733</td>
<td>0.580</td>
<td>6.40</td>
<td>6</td>
</tr>
<tr>
<td>HMS15</td>
<td>1</td>
<td>0.781</td>
<td>0.279</td>
<td>27.47</td>
<td>7</td>
</tr>
<tr>
<td>HMS7</td>
<td>1</td>
<td>0.792</td>
<td>0.694</td>
<td>21.43</td>
<td>7</td>
</tr>
<tr>
<td>LEX34</td>
<td>5</td>
<td>0.723</td>
<td>0.664</td>
<td>3.24</td>
<td>5</td>
</tr>
<tr>
<td>AHT5</td>
<td>8</td>
<td>0.744</td>
<td>0.663</td>
<td>3.50</td>
<td>6</td>
</tr>
<tr>
<td>COR069</td>
<td>13</td>
<td>0.720</td>
<td>0.631</td>
<td>8.75</td>
<td>5</td>
</tr>
<tr>
<td>COR002</td>
<td>14</td>
<td>0.666</td>
<td>0.571</td>
<td>3.35</td>
<td>4</td>
</tr>
<tr>
<td>AHT2</td>
<td>15</td>
<td>0.751</td>
<td>0.614</td>
<td>6.28</td>
<td>4</td>
</tr>
<tr>
<td>B-8</td>
<td>15</td>
<td>0.736</td>
<td>0.623</td>
<td>7.95</td>
<td>8</td>
</tr>
<tr>
<td>LEX056</td>
<td>16</td>
<td>0.837</td>
<td>0.748</td>
<td>7.02</td>
<td>7</td>
</tr>
<tr>
<td>COR032</td>
<td>17</td>
<td>0.454</td>
<td>0.254</td>
<td>3.50</td>
<td>3</td>
</tr>
<tr>
<td>LEX36</td>
<td>19</td>
<td>0.725</td>
<td>0.632</td>
<td>4.40</td>
<td>9</td>
</tr>
<tr>
<td>A-17</td>
<td>26</td>
<td>0.834</td>
<td>0.777</td>
<td>4.75</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 2. Pedigree for mapping the HERDA locus and ECA1 LOD analysis. (A) Pedigree of 11 affected horses (shaded black) that demonstrate the linebreeding used in breeding programs. DNA from 41 unaffected relatives (shaded gray) and all affected horses were used for LOD analysis. (B) Plot of LOD scores for nine markers distributed across ECA1; the maximum score of 7.43 was associated with microsatellite marker AHT58.
Fig. 3. Comparative genomics and reduction of the critical interval containing the HERDA locus. (A) Genetic map of ECA1 [11] and position of nine markers used to broadly map the locus. (B) Blocks of homozygosity found in HERDA-affected horses using microsatellite markers. Six of the microsatellites (1CA25 thru UCDEQ440) that comprise the HERDA ancestral haplotype (hash-marked bar) comprise a radiation hybrid group [12] and indicate the relative order of these markers. Numbers below bars represent the number of affected samples sharing genomic blocks that are identical by descent. (C) Physical map of HSA15q used to develop gene-based markers within equine homologues of human genes. The most significant (Supplemental Table 1) cross-species discontinuous MegaBLAST hit is shown for equine microsatellites to the left. Genes from HSA15q in which informative SNPs were developed from equine homologues are shown to the right. (D) Fine-structure blocks of homozygosity found in HERDA-affected horses using novel SNP and microsatellite markers. Numbers below bars represent the number of affected horses that share genomic blocks that are identical by descent.
ECA1 was confirmed. The other two horses had been euthanized and no further analysis was possible. Horses are subject to a number of dermatological conditions and novel cases which mimic aspects of HERDA may have entered our dataset. These cases highlight the importance of having an accurate way to diagnose HERDA.

BLAST results (Supplemental Table 1) of 9 of the 10 microsatellites used to refine the area of homozygosity within affected samples established a sequence-based framework for comparing the equine and human genomes. Recent comparative maps [11–13] demonstrate the region containing the HERDA locus on ECA1q to be homologous to HSA15q. The highest BLAST hits of the 9 microsatellites span HSA15, between 36 Mb (UM026) and 75.49 Mb (1CA25). The relative order of markers 1CA25, UM043, HTG12, AHT58, UM004, and UCD440 as they BLAST (Fig. 3C) to the human genome correlates with the order of the markers as they appear on the first-generation whole-genome radiation hybrid map (Fig. 3B) [12].

The general region of HSA15 was screened for genes associated with EDS or related genes which were deemed candidate genes based on similarities of phenotype. Human lysyl oxidase like-1 (LOXL1) resides on HSA15q24 (71.94 Mb) and is involved in the metabolism of collagen and elastin. Sequencing of exon 1 from the equine LOXL1 in an affected and unaffected Quarter Horse revealed a c.511C>G SNP that is predicted to cause a glutamic acid to glycine change (p.6E>G) in the putative endoplasmic reticulum (ER) signal sequence, was homozygous (PPIB*1/+, 3 of 16) and heterozygous (PPIB*1/+; 6 of 16) in unaffected horses, indicating that it is not causative for HERDA. PPIB*HRD (c.115G>A) (Fig. 4A inset), predicted to cause a glycine to arginine change (p.39G>R) in the putative N-terminal domain of the protein following ER signal cleavage, was not found in any of the 16 unaffected horses tested. Seven affected horses were homozygous (PPIB*HRD/PPIB*HRD) and heterozygous (PPIB*1/+; 6 of 16) in unaffected horses, and is involved in the metabolism of collagen and elastin.

Table 2
Informative genetic markers for refining the critical interval surrounding the HERDA locus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Ta (bp)</th>
<th>Size (bp)</th>
<th>Sequencing primer</th>
<th>Bases from end</th>
<th>SNP w/ flanking sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNL2</td>
<td>F: CTGTCAGAAAATCATGACTTCTTCR</td>
<td>61</td>
<td>948</td>
<td>R</td>
<td>155</td>
<td>Control: GGAGAAA CCACAAGC</td>
</tr>
<tr>
<td></td>
<td>R:CAGACTCCCACCCCAAGATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HERDA: GGAGAACCTCACAAGC</td>
</tr>
<tr>
<td>USP3</td>
<td>F: AGCTTTACAAGCTGACAGGACATA</td>
<td>60</td>
<td>1640</td>
<td>F</td>
<td>340</td>
<td>Control: AACACCCCTCAGTGT</td>
</tr>
<tr>
<td></td>
<td>R: TGAGGACTGAAAGGAGGACTCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HERDA: AACACCCCTCAGTGT</td>
</tr>
<tr>
<td>SPG21</td>
<td>F: VIC-TGACTACGCTAGCCCCGTGATCR</td>
<td>58</td>
<td>224–228</td>
<td>N/A</td>
<td>N/A</td>
<td>Control: 224, 226, 228</td>
</tr>
<tr>
<td></td>
<td>R: TTATATTTTCTTCCTCCTCAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HERDA: 226</td>
</tr>
<tr>
<td>CILP</td>
<td>F: TGAGCCCTCAACAGGGAGGACACR</td>
<td>62</td>
<td>1394</td>
<td>R</td>
<td>154</td>
<td>Control: AGACAGGGTTTAATGT</td>
</tr>
<tr>
<td></td>
<td>R: CACTTGCTCCAGGGAGGACAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HERDA: AGACAGGGTTTAATGT</td>
</tr>
<tr>
<td>ITGA11</td>
<td>F: ACCTGTCTCCTGCGGCCCTC</td>
<td>59</td>
<td>1554</td>
<td>R</td>
<td>79</td>
<td>Control: CGGTCCCCGGCTCTCA</td>
</tr>
<tr>
<td></td>
<td>R: CAGCAGATGGAAGTGTGATCCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HERDA: CGGTCCCCGGCTCTCA</td>
</tr>
<tr>
<td>LOXL1</td>
<td>F: CGCACCTCGGTCTCCACG</td>
<td>60</td>
<td>404</td>
<td>F</td>
<td>85</td>
<td>Control: CCAGCCCCGGCTCTCTTT</td>
</tr>
</tbody>
</table>

Mutation screen

The HERDA locus maps within a block of synteny that has been conserved across human, chimpanzee, rhesus monkey, dog, mouse, and rat. The region, which is typically bounded by TNL2 and SPG21, is predicted to contain 20 known genes and 6 putative genes in humans (Supplemental Table 2). Additional research into the functions of genes in the block led to the sequencing of equine PPIB based on its peptidyl-prolyl cis-trans isomerase function, a published association with procollagen [14–16], and the role of extracellular cyclophilins in the inflammatory response [17].

Two SNPs predicted to cause missense mutations were found in PPIB by sequencing cDNA (GenBank Accession No. EF397503) from skin of an affected and an unaffected horse. All four introns of PPIB were sequenced and no SNPs were found. PPIB*1 (c.17A>G), predicted to cause a glutamic acid to glycine change (p.6E>G) in the putative endoplasmic reticulum (ER) signal sequence, was homozygous (PPIB*1/+, 3 of 16) and heterozygous (PPIB*1/+; 6 of 16) in unaffected horses, indicating that it is not causative for HERDA. PPIB*HRD (c.115G>A) (Fig. 4A inset), predicted to cause a glycine to arginine change (p.39G>R) in the putative N-terminal domain of the protein following ER signal cleavage, was not found in any of the 16 unaffected horses tested. Seven affected horses were homozygous (PPIB*HRD/PPIB*HRD), eight unaffected relatives were heterozygous (PPIB*HRD/+), and a single unaffected full-sibling was homozygous for the wild-type SNP (+/+) .

An assay was developed (Fig. 4A) so that large numbers of samples could be screened for the presence of PPIB*HRD. All 64 HERDA samples that contained a block of homozygosity...
including AHT58 (Fig. 3B) were homozygous (PPIB*HRD/PPIB*HRD) for the mutation (Table 3). None of the 4 genetically distinct horses that had previously been flagged as potential misdiagnosed cases carried the HERDA allele (+/+, 4 of 4). Thirty-four available parents of affected horses were heterozygous (PPIB*HRD/+), consistent with the autosomal recessive nature of the disease. A $\chi^2$ test of 13 genotyped trios indicates that the expected number of affected horses with a PPIB*HRD/PPIB*HRD genotype (3.25) is significantly different ($p<0.001$) from that observed (13). Using binomial probability, the likelihood of all 13 affected horses being homozygous for PPIB*HRD is $p=1.5 \times 10^{-8}$.

Previous pedigree analysis of the families used in establishing a LOD score for the HERDA locus display an inbreeding
loop which represents the most likely path of the transmission of the mutation. In all cases, PPIB*HRD segregates in a predictable fashion consistent with the hypothesis that inbreeding is leading to the union of two mutant alleles which are identical-by-descent. A set of 1079 unaffected Quarter Horses collected from the UC Davis Veterinary Medical Teaching Hospital and the UC Davis Veterinary Genetics Laboratory (VGL) were screened for the mutation (Table 3). Thirty-eight samples were heterozygous for the mutation, suggesting a 3.5% carrier frequency, which agrees with previous estimates of 1.8 to 6.5% [2]. No control Quarter Horses were detected to be homozygous for the mutation from this sample set. A small sampling of Arabians (18), Paint Horses (28), and draft horses (9) were tested and only the wild type allele was detected.

The HERDA-predicted PPIB protein and one of the two equine wild-type variants were aligned with five mammalian and three nonmammalian vertebrate (Danio rerio, Xenopus tropicalis, and Gallus gallus) PPIB sequences (Fig. 4B). Equine PPIB shared the highest identity (97.7%) with canine PPIB. The six mammalian PPIB sequences were 88% identical. Across sequenced vertebrates, the glycine residue that is mutated in HERDA horses is invariant. The glycine sits in the third position of a completely conserved seven-amino-acid peptide (37KKGPKVT43) structure that has been strictly maintained throughout vertebrate evolution.

**PPIB as a compelling candidate causal mutation for HERDA in the American Quarter Horse**

The detection of unique polymorphisms in the identical-by-descent critical interval was a first step toward developing a definitive genetic test for HERDA. Of the 64 affected horses which share the characteristic HERDA haplotype across ECA1q, four DNA markers have been determined to be homozygous in all samples: the A→G intronic SNP of USP3 (Table 2), the c.17A→G exonic SNP (PPIB*1) predicted to cause a missense mutation of PPIB, the c.115G→A exonic SNP (PPIB*HRD) predicted to cause a missense mutation of PPIB, and the 185 allele of the AHTS8 microsatellite marker. Of these four markers, the c.115G→A missense mutation in PPIB is the only polymorphism unique to families segregating for HERDA and only affected horses were homozygous. Equine cDNA sequence from an additional 12 genes (GenBank Accession Nos. EF397504–EF397516) predicted in the block of identity-by-descent have been interrogated and no SNPs have been found in coding sequence.

**PPIB** is a member of the peptidyl-prolyl isomerase (PPI) gene family. Human and mouse genomes each have eight PPI family members (PPIA–PPID), which contain a conserved active domain and alternative N- and C-terminal domains specifying the individual PPI family member. This family of genes, of which PPIA is the most comprehensively studied, is implicated in protein folding, immune response via its binding of cyclosporine A, and T cell activation. Although naturally occurring PPI mutants have not been reported, a mouse PPIA knockout was shown to be viable and 30% of ppia -/- mice developed a progressive form of blepharitis, or inflammation of the eyelids, beginning at 3 months of age and elevated Th2 cytokine production [18]. The fact that PPI deficiencies are tolerated biologically and produce an adult-onset inflammatory phenotype supports PPIB as a causal candidate mutation for HERDA.

PPIs have been implicated in protein folding of collagens via their *cis-trans* peptidyl-prolyl isomerase function [14–16]. Structural collagens contain a high percentage of proline residues (average 18% in humans) which are necessary for the proper formation of collagen fibrils. Kinetic studies of collagen folding indicate two steps in fibril formation, the slower of which is limited by the *cis-trans* isomerization of proline residues in the collagen chain [19]. Histological examination of affected tissue has consistently shown disorganization of collagen. While it remains unclear whether deficient collagen folding is related to the development of severe seromas and ulcerations, PPIB is a logical candidate gene that would explain a portion of the histological phenotype observed in affected HERDA tissue.

Work on PPIB has clearly demonstrated two important binding sites: type I corresponding to functional receptors and type II corresponding to sulfated glycosaminoglycans [20]. Site-directed mutagenesis experiments demonstrated that T-lymphocyte adhesion to the extracellular matrix, specifically fibronectin, is dependent on the ability of PPIB to bind glycosaminoglycan [21,22]. Three lysine residues in the N-terminal domain of PPIB are essential for heparin sulfate binding and consequently appropriate T-lymphocyte adhesion. The mutated p.39G>R seen in HERDA horses is immediately downstream of the lysine triplet 36KKK38 (Fig. 4B). Based on this cumulative empirical evidence, one hypothesis is that mutation of the compact glycine residue into a large, charged arginine introduces steric hinderance, resulting in altered heparin sulfate binding. Complicating our elucidation of the effects of this p.39G>R mutation on PPIB function is the fact that arginine, which is similar in size and charge to lysine, has been shown to have a higher affinity for heparin than lysine [23]. Due to the multiple functions associated with PPIB and the poorly characterized pathways that lie downstream of the protein with regard to intercellular signaling [24], it is difficult to predict how a mutation in this gene would manifest itself biologically.
Population genetics and DNA testing

The increase in HERDA cases during the late 1990s is intriguing from a population genetics perspective. Similar to many other domesticated animal species, Quarter Horse breeders commonly use linebreeding to select for desirable qualities. While the breed can be used for a wide variety of recreational activities, pedigree analysis of HERDA horses suggests that the disease is concentrated within particular lines of cutting horses. Cutting is a term used to describe the process of singling out and isolating an individual from a herd of cattle and maintaining that separation, as would be practical on a ranch. Horses require training to hone their skills and speed, agility, and “cow sense” are paramount to mastering the art of cutting. Competitions were begun in 1898 and have grown considerably during the past century, with National Cutting Horse Association approved events offering total purses exceeding $39 million as of 2006. Success at cutting events and the money which accompanies that success has consequently had a profound impact on the selection of breeding stallions.

A number of obligate carriers have contributed disproportionately to the breeding population of Quarter Horses within the past 2 decades. Inbreeding loops evident in many of the affected horses’ pedigrees trace back to these popular sires and invite the hypothesis that a heterozygous genotype may confer an advantage as it relates to cutting. Whether PPiB*HRD or the larger haplotype associated with the allele has been under selection remains unclear. Further functional work would be required to establish such a heterozygous advantage. Regardless, robust breeding of carrier stallions has likely led to an increase in the allele frequency and the use of linebreeding has resulted in a higher incidence of affected HERDA cases. The PPiB*HRD allele which has been found in perfect association with the HERDA phenotype will be useful to Quarter Horse breeders for two reasons. First, in cases where a young horse begins to show skin irregularities, the test will give owners the chance to genetically determine whether HERDA is the cause and prevent a potentially unnecessary euthanasia. Second, it will allow breeders the opportunity to identify carriers within the breeding population and minimize the production of affected HERDA horses.

Materials and methods

DNA and tissue samples

Diagnosed cases of HERDA and control samples were collected as previously reported [2]. With the permission of the American Quarter Horse Association (AQHA), backlogged hair root samples of relatives of affected horses were made available from the VGL, which conducts parentage testing for all registered American Quarter Horses. Genomic DNA was isolated from blood samples using the Qiagen Blood Mini-Kit (Qiagen, Valencia, CA). Genomic DNA was isolated from hair root samples by the VGL [8]. The sample set consisted of 68 horses diagnosed with HERDA, 76 unaffected relatives, 1079 Quarter Horses to serve as controls, and 55 horses of diverse heritage (Arabians, Paint Horses, and draft horses).

Microsatellite genotyping

Most fluorescently labeled primer sets were obtained with the help of the Dorothy Havemeyer Foundation. Primers for additional published microsatellite markers were obtained from Applied Biosystems (Foster City, CA). Multiplex reactions for the initial genome scan were based on previous reports [8] and personal communications with the VGL. Data for 27 of the 100 loci screened in the genome scan were generated by the VGL as part of their standard parentage panel and genotyped in three multiplex reactions. Sixty-seven additional markers were combined into 14 multiplex reactions. Seven microsatellites were individually amplified. Amplifications were performed in 25 μL total volume containing 1 μL genomic DNA (~20 ng), 1× PCR buffer, 2.5 mM MgCl2, 250 μM dNTPs, 1 unit AmpliTaq Gold (Applied Biosystems) with primer concentrations and annealing temperatures specified in Supplemental Table 3. For fine-structure mapping, markers spanning ECA1 were amplified in 20 μL total volume containing 1 μL genomic DNA, 1× PCR Buffer, 1.5 mM MgCl2, 125 μM dNTPs, 0.5 units AmpliTaq Gold with primer concentrations and annealing temperatures specified in Supplemental Table 4. All microsatellite data was analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems) and STRAND software (http://www.vgl.ucdavis.edu/STRAND/).

Statistics

Allele and genotype frequencies were counted within the control population (n=44) and the affected population (n=38) at 98 autosomal microsatellite loci. For each group, the expected heterozygosity values with standard errors and observed heterozygosity values of each locus were computed with Arlequin software [25]. A χ2 test assigned p values by testing the null hypothesis that the two population samples have the same allele frequencies. An 1×2 contingency table with 1-L degrees of freedom, where L=No. of alleles present at a given locus, were used. LOD scores were generated using HOMOZ/MAPMAKER software [26].

Establishing a comparative framework

Published microsatellite sequence from ECA1q4 was compared to the Equus caballus whole-genome sequence (WGS) Trace Archives using Discontinuous MegaBLAST (http://www.ncbi.nlm.nih.gov/blast/traceemb.shtml). Five hundred bases were mined 5′ and 3′ to the polymorphic dinucleotide repeat to generate >1000 continuous bases of equine sequence. Those sequences which contained informative equine genetic markers were compared to the human genome sequence (Build 36.2) using BLAST (http://www.ncbi.nih.gov). A significant similarity match between equine and human sequences was set at an alignment score (S)=60 and a sum probability value (E)<3.0 E-06, similar to a previous report [27]. Subsequently, the region of the human genome identified with BLAST comparisons was compared with other fully and partially mapped mammalian genomes using the UCSC Genome Browser to confirm conservation of synteny across species (http://genome.ucsc.edu). A list of equine candidate genes was generated from the region of synteny from the human genome (Build 35.1). The Horse Genome Project (http://www.thio-hannover.de/einricht/zucht/hgp/index.html) was searched for equine BAC clones which had both ends successfully sequenced and that BLAST within 250 kb of each other on HSA15, surrounding the region of identity by descent [28]. These BAC clones provided additional confidence in the physical relationship between ECA1 and HSA15 and helped to verify our candidate gene list. Equine gene-specific markers were optimized with the 5000 rad equine radiation hybrid (RH) panel and submitted for analysis [12].

SNP discovery and genotyping

Genes for SNP discovery were selected based on their spacing and the availability of mammalian mRNA sequences from the GenBank database. Sequences from all available mammals (typically human, mouse, dog, and cow) were aligned (Vector NTI) and searched for regions of high conservation across species. In addition, human and mouse mRNA sequences were analyzed using the BLAT function of the UCSC genome browser (http://genome.ucsc.edu) to determine intron/exon boundaries and the trend in intron size. Introns which had conserved sizes between 700 bp and 3 Kb were targeted to facilitate cloning and sequencing. These features were used to design primers for amplification of specific homologous sequences from the horse genome.

Genomic DNA from an unaffected and an affected Quarter Horse were used to amplify corresponding genomic fragments for each of the genes in Table 2.
Fragments were cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), sequenced, and verified by BLAST analysis to ensure that both ends of cloned exonic sequence were indicative of the targeted gene. Clones were aligned to identify gene-specific intronic SNPs or microsatellites. To genotype SNPs from additional affected horses and unaffected controls, genomic fragments were amplified, purified with the Qiaquick Purification Kit (Qiagen, Valencia, CA), and sequenced on an ABI 3100 Genetic Analyzer with one of the gene-specific primers used in the original amplification. The polymorphic microsatellite within the intron of SPG21 was genotyped using a fluorescently labeled primer as previously described.

Sequencing of candidate genes

Skin fibroblasts derived from an affected HERDA horse and an age-matched unaffected Quarter Horse were used to generate cDNA libraries (Fast Track 2.0 mRNA Isolation Kit, Invitrogen; Marathon cDNA Amplification Kit, BD Biosciences). 5′ and 3′ RACE reactions were carried out with appropriate reverse and forward primers (Supplemental Table 5), separated by gel electrophoresis, extracted, and sequenced directly to obtain coding and partial 5′UTR and 3′UTR. For PPIB, additional primers were designed to generate sequence of the four predicted introns (Supplemental Table 5). When equine whole-genome sequence trace archives became available (spring 2006), equine homologues of human genes from the syntenic region were computationally mined. Discontinuous MegabLAST was used to design primers that generated maximal equine coding sequence. cDNA products were amplified, cloned, and sequenced to identify additional SNPs between the affected and the unaffected cDNA libraries.

DNA testing

Primers were designed to amplify a fragment of the PPIB gene, which contains an informative SNP, from equine gDNA. An unlabeled forward primer (5′CGGTTGAGTGCTCCTTTC) and a fluorescently labeled reverse primer (5′6FAM-GCCCAAGGCCAGCTCTAGGA) generate a 250-bp fragment that is susceptible to Earl restriction endonuclease digestion following amplification. An Earl site cuts 46 bp from the end of the forward primer and serves as an internal control to verify that the enzyme is working properly. The informative SNP detected in the HERDA population introduces a second Earl site that cuts 67 bp from the first Earl site.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2007.03.009.

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