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Investigation of Single Nucleotide Polymorphisms Located On 5 Candidate Genes and Their Association with Tendon Injuries in a Population of Multi-Discipline Trained Horses

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Abstract

Tendon and ligament injuries (TLIs) in the performance horse represent a significant burden to the equine industry. Prevention of these types of injury is a major goal as treatment is often unsuccessful and re-injury common. The objective of the current study was to evaluate a population of athletically trained horses from multiple disciplines for SNP located five candidate genes in association TLIs. A population of 63 performance horses with documented injury history or injury resistance was utilized for the current study. Specifically, the study was comprised of 25 horses of various ages with at least one TLI injury and 33 horses of various ages with no injury history. The five candidate genes selected for evaluation included the Angiotensin 1 converter enzyme gene (ACE), the ATPase alpha 2 peptide gene (ATP1A2) the Bradykinin receptor B2 gene (BDKRB2), the Collagen type 1 alpha 1 gene (COL1A1) and the Collagen type 5 alpha 1 gene (COL5A1). Candidate genes in the current study have been previously reported to be associated with jumping ability (ACE), racing ability (ATP1A2), and ligament and tendon injuries (BDKRB2, COL1A1 and COL5A1). A total of 64 single nucleotide polymorphisms (SNP) were selected across all candidate genes (ACE = 11, ATP1A2 = 14, BDKRB2 = 9, COL1A1 = 14, COL5A1 = 16). A mixed model analysis was utilized with independent variables of breed, sex, discipline, and individual SNP marker genotype being analyzed as sources of variation for TLI's. Dependent variables included probability of TLI during the horses lifetime (injured or not injured during lifetime) and age of first TLI injury. Although multiple SNP were significantly associated with probability of lifetime injury and age of first TLI injury in the current study, these SNP and a greater number of candidate genes must be evaluated in larger more diverse populations prior to implementation into selection strategies.

Keywords: *Equine; Injury; SNP; Candidate Genes*

Introduction

Evaluation of single nucleotide polymorphisms (SNP's) located on candidate genes and QTL regions to analyze potential associations with economically important traits in livestock has become a major area of research. Specifically, SNPs from genomic regions have been associated with milk production [1], meat quality [2], fertility, [3], and growth and performance [4] in livestock. However, very limited genomic research has been conducted in the equine industry in relation to TLI (tendon and ligament injuries) predisposition in athletically trained horses from all disciplines. The completion of the horse genome mapping project in 2009 [5] and candidate genes previously described in the human genome with sports ability/injury make identification of SNP significantly associated with TLI injuries in the horse industry a reality. However, while many studies conducted in humans have identified candidate genes associated with injury predisposition [6-8] these genes currently have no SNP annotated on the equine genome sequence. When evaluating athletic performance, the Angiotensin 1 converter enzyme gene (ACE), Bradykinin receptor B2 Gene (BDKRB2) and the ATPase alpha 2 peptide gene (ATP1A2)

have been associated with traits such as explosive muscle strength in human soccer players [9] jumping ability in human athletes [10], athletic endurance in humans [11] and also have SNP annotated on them from the equine genome.

Multiple genes have been reported to be associated with injury predisposition in humans as well. The BDKRB2 gene, the Collagen type 1 alpha 1 gene (COL1A1) and the Collagen type 5 alpha 1 gene (COL5A1) have all been associated with tendon/ligament injuries [8,12] as well as arthritis [13,14] in human studies. The COL5A1 gene has further been reported to be significantly associated with tendon injuries in horses [15]. Tully and associates identified two SNP located on the COL5A1 gene in which horses inheriting specific SNP genotypes were less likely to have superficial digital flexor tendon injuries than individuals inheriting other genotypes at the same SNP locus.

Objective

The objective of the current study was to evaluate SNPs located on five equine candidate genes that have previously described in the human and equine genomes to influence athletic performance and injury predisposition for their potential associations with TLI injuries in performance horses.

Materials and Methods

Experimental Animal population

All animals were treated and samples collected in accordance with the principles and guidelines outlined in the Guide for the Care and Use of Agricultural Animals in Research and Teaching. Data from a population of 62 Louisiana performance horses was collected through an owner generated injury history survey. Specifically, the study was comprised of 25 competition horses of various ages with at least one TLI injury and 33 competition horses of various ages with no injury history. The various competition disciplines that comprised the research population included racing, dressage, jumper, cutting, and horses used for more than one of the previously described disciplines. The data included, age, sex, breed, age at first training, type of discipline, age of first competition number of years in competition, age of first TLI and overall TLI status during the horses lifetime (injured vs no-lifetime injury).

DNA Extraction

A 10ml blood sample was collected via jugular venipuncture and stored in an anti-coagulating solution. The sample was centrifuged in a high speed refrigerated centrifuge to separate red blood cells, and white blood cell buffy coats. The white blood cell buffy coats were utilized for DNA extraction and DNA was extracted utilizing a previously described saturated salt procedure described by Miller and associates [16]. DNA concentration was measured after extraction and stored at -20 degrees until marker analyses were conducted.

SNP Selection and Analysis

SNP located on candidate genes were selected for the current study utilizing the dbSNP function of the NCBI database (http://www.ncbi.nlm.nih.gov/snp). Due to the varying amount of annotation in the equine genome, all the SNP sequence that was available for the horse was selected for genotyping in the current study (Table 1). Single nucleotide genotypes were generated utilizing a Sequenom genotyping platform (Sequenom Laboratories, San Diego, CA). Genotyping of the SNP in the current study was conducted by the NeoGene Corporation (Lincoln, NE).

Statistical Analyses

The mixed model procedure of SAS (version 9.4) was utilized to analyze potential associations of SNPs to TLI injury in the current population. Significance in the current study was set at $P < 0.1$ due to the small experimental population and the novel nature of the study. Independent variables fit in the model included, breed, sex, discipline and SNP genotype. Dependent variables fit in the statistical model included age of first TLI, and probability of TLI during the horse's lifetime (injured or not injured during lifetime). The LSMEANS function along with the pre-planned pair wise comparison function of SAS will be utilized to determine if inheritance of differing genotypes led to significantly ($P < 0.1$) greater or reduced probability and age of TLI in multi-discipline completion horses.

Gene Name	Gene Symbol	Number of SNPs Genotyped	Associated Traits
Angiotensin 1 converter enzyme gene	ACE	11	Jumping ability/Muscle power
ATPase alpha 2 peptide gene	ATP1A2	14	Performance endurance
Bradykinin receptor B2 gene	BDKRB2	9	Explosive muscle strength/Arthritis
Collagen type 1 alpha 1 gene	COL1A1	14	Tendon and ligament injury
Collagen type 5 alpha 1 gene	COL5A1	16	Tendon, ligament injury/Arthritis

Table 1: *Candidate genes utilized in the current study and the number of SNP genotyped per gene.*

Results and Discussion

The evaluation of SNP located on five candidate genes in the equine genome revealed multiple SNP that were significantly ($P < 0.1$) associated with probability of TLI and age of first TLI. When evaluating the trait of age of first TLI, 11 unique SNP from three candidate genes were significantly ($P < 0.1$) associated (Table 2). However, for every marker that was identified as significant, breed was also a significant source of variation in the statistical model ($P < 0.1$). This was probably due to the majority of the population utilized herein being made up of Thoroughbreds and Quarter horses, with the Thoroughbreds having the highest frequency of injured individuals. Animals inheriting the heterozygous genotype from SNP's rs394325220 rs395082490 located on the ATP1A2 gene and SNP rs395958795 located on the COL5A1 gene had an TLI significantly ($P < 0.1$) earlier in their lifetime than animals inheriting the homozygous major allele genotype. However, the opposite effect was observed for SNP rs395396934, rs397343516, rs396901617, and rs397378257 located on the ATP1A2 gene and SNP rs394134479 located on the BDKRB2 gene in which animals inheriting the homozygous major allele genotype from these SNP experienced a TLI significantly earlier in age than animals inheriting the heterozygous genotype. Individuals inheriting the minor allele homozygous genotype for SNP rs69261397, and rs393921295 located on the COL5A1 gene had significantly earlier TLIs than individuals inheriting the major allele genotypes. However, animals inheriting the minor allele homozygous genotype from SNP rs395524081 located on the COL5A1 gene had a TLI much later in life than animals inheriting the heterozygous or major allele homozygous genotypes.

SNP ID	Gene	*P* Value	SNP	Minor Allele Genotype	Heterozygous Genotype	Major Allele Genotype
rs394325220	ATP1A2	0.08	A/C		$9.23 + 0.72^{a}$	$13.73 + 2.12^{b}$
rs395082490	ATP1A2	0.03	C/T		$7.44 + 1.02^{a}$	$10.38 + 0.69^{b}$
rs395396934	ATP1A2	0.08	A/G		$12.61 + 1.61^{a}$	$8.11 + 1.01^{b}$
rs396901617	ATP1A2	0.08	C/G		$13.73 + 2.12^{a}$	$9.23 + 0.72^{b}$
rs397343516	ATP1A2	0.08	C/G		$13.73 + 2.12^{a}$	$9.23 + 0.72^{b}$
rs397378257	ATP1A2	0.03	G/T		$11.11 + 0.84^{a}$	$8.18 + 0.82^{b}$
rs 394134479	BDKRB2	0.06	A/G		$12.59 + 1.49^{a}$	$8.42 + 0.86^{b}$
rs69261397	COL5A1	0.06	C/T	$7.50 + 1.00^{a}$	$14.17 + 1.63^{b}$	$12.00 + 1.26^{b}$
rs393921295	COL5A1	0.1	A/G	$4.36 + 2.23^{a}$	$7.36 + 2.24^{ab}$	$10.36 + 0.76^{b}$
rs395524081	COL5A1	0.08	A/G	$11.52 + 1.04^{a}$	$9.60 + 0.87^{b}$	$5.10 + 1.85^{b}$
rs395958795	COL5A1	0.09	T/C		$7.29 + 1.37^{a}$	$10.20 + 0.77^{b}$

Table 2: *Single nucleotide polymorphisms significantly associated with age of injury and LSMEANS estimate comparisons between reported genotypes.*

While the results presented herein for age of first TLI are in agreement with previous reports of the COL5A1 gene being associated with tendon and ligament injuries [15,17], the current study is the first to report significant SNP located on the COL5A1 gene in the equine species significantly associated with age of first TLI. Furthermore, this is the first study to identify SNP located on the ATP1A2 gene and the BDKRB2 genes associated with TLI in the equine species as these genes have previously only been described with sports performance in humans [9,11] and osteoarthritis [14].

When evaluating the trait of probability of lifetime TLI, analyses revealed 7 unique SNP from four candidate genes that were significantly ($P < 0.1$) associated with this trait. However, breed was once again a significant source of variation ($P < 0.1$) in the statistical model. Specifically, one SNP from the ACE gene, one SNP from the COL5A1 gene, three SNP from the ATP1A1 gene and three SNP from the COL1A1 gene were identified as significant (Table 3). Animals inheriting the heterozygous genotype from SNP's rs394071979 located on the ACE gene and SNP's rs396920469 and rs395905364 located on the COL1A1 gene had a significantly ($P < 0.1$) lower probability of lifetime TLI than animals inheriting the major allele homozygous genotype. Individuals inheriting the homozygous minor genotype from SNP rs397235747 and rs395575417 located on the ATP1A1 gene had significantly lower probability of lifetime TLI than those individuals inheriting the heterozygous genotypes, whereas, individuals inheriting the heterozygous genotype from SNP rs396366759 had significantly higher probabilities of lifetime TLI than individuals inheriting the major homozygous allele genotype. Only one SNP from the COL5A1 gene was significantly associated with probability of a lifetime TLI. Individuals inheriting the major and minor homozygous genotypes had a significantly higher probability ($P < 0.1$) of TLI than individuals inheriting the heterozygous genotype.

SNP ID	Gene	P Value	SNP	Minor Allele Genotype	Heterozygous Genotype	Major Allele Genotype
rs394071979	ACE	0.06	T/G		0.02^{a}	$0.21 + 0.15^{b}$
rs397235747	ATP1A1	0.1	T/C	$0.07 + 0.20^{a}$	$0.42 + 0.16^{b}$	$0.11 + 0.19^{a}$
rs395575417	ATP1A2	0.03	A/G	$0.03 + 0.18^{a}$	$0.49 + 0.16^{b}$	$0.19 + 0.19^{ab}$
rs396366759	ATP1A2	0.09	G/T	$0.25 + 0.25^{ab}$	$0.52 + 0.16^{a}$	$0.08 + 0.20^{b}$
rs396920469	COL1A1	0.08	G/C		$0.11 + 0.25^{a}$	$0.34 + 0.15^{b}$
rs395905364	COL1A1	0.08	T/C		$0.12 + 0.25^{a}$	$0.34 + 0.15^{b}$
rs393921295	COL5A1	0.07	A/G	$0.72 + 0.53^{a}$	$0.09 + 0.21^{b}$	$0.36 + 0.15^{a}$

**Superscripts differ at $P < 0.05$ within row.*

Table 3: *Single nucleotide polymorphisms significantly associated with lifetime injury status and LSMEANS estimate comparisons between reported genotypes.*

While 7 unique SNP were identified in the current study only one SNP identified on the COL1A1 and COL5A1 genes validate previous studies reporting these genes as being associated with TLI predisposition. However, only one of these studies, (Tully et al., 2014) reported the COL5A1 genes as being associated with injury in horses in contrast to previous work that has described these genes being associated with injury predisposition in humans [8,12]. Furthermore, SNP located on both the ACE and ATP1A1 genes were reported as significantly ($P < 0.1$) affecting probability of a lifetime TLI. The current study is the first to report SNP in the equine genome significantly associated with lifetime TLI predisposition as previous work has described the ACE and ATP1A1 genes as being associated with athletic performance in humans and not injury [9,10,13].

Conclusion

Although the current study identified multiple SNP located on 5 candidate genes, these SNP must first be validated in larger much more diverse populations prior to implementation into selection strategies or breeding decisions. Increasing the experimental population size has the potential to properly develop and validate SNP identified in the current study as being significantly associated with TLI

predisposition. If properly validated, these SNP have the potential to make a significant impact in the modern horse industry through the early identification of TLI prone individuals. Identification of more SNP on more candidate genes in the horse genome may not only allow for the early identification (immediately after birth) of animals predisposed for injury predisposition but it would allow for these animals to be trained/managed more effectively. Furthermore, it would allow the horse industry to be more accurate in their mating pairings through the identification of optimal breeding individuals not only for performance but to also decrease genetic predisposition to injury in the offspring.

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