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The Candidate Gene *XIRP2* at a Quantitative Gene Locus on Equine Chromosome 18 Associated with Osteochondrosis in Fetlock and Hock Joints of South German Coldblood Horses

Catherine Wittwer, Henning Hamann, and Ottmar Distl

From the Institute for Animal Breeding and Genetics, University of Veterinary Medicine Hannover, Foundation Bünteweg 17p, 30559 Hannover, Germany.

Address correspondence to O. Distl at the address above, or e-mail: ottmar.distl@tiho-hannover.de.

A whole-genome scan for radiological signs of osteochondrosis (OC) and osteochondrosis dissecans (OCD) in South German Coldblood (SGC) horses using 250 microsatellite markers identified a genome-wide significant quantitative trait locus (QTL) for fetlock OCD and a chromosome-wide QTL for hock OC on *Equus caballus* chromosome (ECA) 18 at a relative position of 45.9–78.2 cM. The aim of this study was to analyze associations of single-nucleotide polymorphisms (SNPs) in candidate genes for OC in this QTL region using 96 SGC horses. The OC-QTL on ECA18 could be confirmed and narrowed down to an interval of 13 Mb between *GALNT13* and *Xin actin-binding repeat containing 2* (*XIRP2*). SNPs in the *XIRP2* gene were significantly associated with fetlock OC, fetlock OCD, and hock OC. The significant associations of SNPs in *XIRP2* could be confirmed in linear animal models controlling for systematic environmental and residual quantitative genetic effects. The significant additive genetic effects of the intronic SNPs (AJ885515:g.159A>G, AJ885515:g.445T>C) in *XIRP2* were 0.15 ($P = 0.01$) for fetlock OC, 0.27 ($P = 0.01$) for fetlock OCD, and 0.15–0.16 ($P = 0.01$–0.02) for hock OC. Homozygous (A/A or T/T) and heterozygous horses were at a 1.3- to 2.4-fold higher risk for fetlock and hock OC. These results suggest that dominant variants of *XIRP2* may be involved in pathogenesis of equine OC.

Key words: *association analysis, horse, osteochondrosis, single-nucleotide polymorphisms, XIRP2*

Osteochondrosis (OC) is a disease of the locomotory system that frequently affects young horses (Jeffcott 1991; Stock et al. 2005; Wittwer et al. 2007a). Disturbed differentiation and maturation of growing cartilage primarily at specific predilection sites of articulations predispose horses for OC (Jeffcott and Henson 1998). Most common signs of OC are cartilage flaps, osseous fragments, and synovial effusions (Trotter and McIlwraith 1981; Jeffcott and Henson 1998). Osteochondrosis dissecans (OCD) is a specific advanced manifestation of OC that is characterized as separation of cartilage or osteochondral fragments (joint mice, chips, corpora libera) from the articular surface. In trotters and Coldblood horses, palmar/plantar osteochondral fragments (POFs) are often seen in fetlock joints of front and hind limbs (Philipsson et al. 1993; Wittwer et al. 2006). In South German Coldblood (SGC) horses, POFs and OC were phenotypically and genetically correlated and, thus, POFs may also belong to the OC complex in this breed (Wittwer et al. 2007a).

Due to the welfare and economic impact of OC, genome mapping efforts in horses aimed to identify genes responsible for this disease. Recently, a complete genome scan for OC in SGC horses had shown quantitative trait loci (QTLs) on horse chromosome (ECA) 18 (Wittwer et al. 2007b). In total, 17 putative QTL on 17 equine chromosomes were identified. These QTLs showed significant effects for fetlock OC on 9 chromosomes, for fetlock OCD on 5 chromosomes, for hock OC on 3 chromosomes, and for OC in fetlock and/or hock joints on 7 chromosomes. Furthermore, 6 QTLs were determined for POFs. On ECA18, a genome-wide significant QTL was located at 45.9 cM for fetlock OCD. A further QTL on ECA18 was shown at 78.2 cM for hock OC and POFs (Wittwer et al. 2007b).

The region between 45.9 and 87.6 cM on ECA18 is syntenic to human chromosome (HSA) 2q14–q32 (Wagner et al. 2006). This homology between ECA18 and HSA2q had also been shown by other reports (Chowdhary et al. 2003; Penedo et al. 2005; Perrocheau et al. 2006; Swinburne et al. 2006). Furthermore, the human–horse synteny was verified employing an in silico analysis of the horse genome assembly (EquCab2.0) including 60 sequence tagged sites (STSs) of 43 equine genes, 16 microsatellites, and a bacterial artificial chromosome end sequence. These STSs were evenly spaced on HSA2q at 136–201.4 Mb and on ECA18 at 26.15–67.02 Mb. The mean distance of the genes selected for development of gene-associated single-nucleotide polymorphisms (SNPs) was approximately 2.25 Mb within this interval on ECA18. We have chosen the genomic region at 45.9–87.6 cM for fine mapping because this region showed one of the highest test statistics among the OC-QTL detected in SGC, and markers in this region were chromosome-wide significant for several signs of OC. As this region on ECA18 was completely syntenic to HSA2q, candidate genes and their location on equine maps could be verified more easily using comparative human–equine maps.

In this syntenic region on HSA2q at 136–201.4 Mb, there are located 5 candidate genes which were reported to cause conditions in man similar to equine OC and/or are expressed in equine cartilage. The 3 candidate genes *frizzled related protein* (*FRZB*), *collagen type III alpha* (*COL3A1*), and *collagen type V alpha 2* (*COL5A2*) were notified by Wittwer et al. (2007b). *FRZB* is involved in osteoarthritis in man and is also expressed in equine cartilage (Loughlin et al. 2004). Mutations in *COL3A1* and *COL5A2* are associated with human Ehlers–Danlos syndrome (Michalickova et al. 1998). Furthermore, the *activin A receptor, type I* (*ACVR1*) gene, a dimeric growth and differentiation factor of the transforming growth factor-beta superfamily, is located in this region. A previous study in man found a common and recurrent mutation in the *ACVR1* (617G>A, R206H) gene, which causes inherited and sporadic fibrodysplasia ossificans progressiva (FOP) in the global population (Nakajima et al. 2007; Shore et al. 2006). The R206H mutation causing FOP is one of the most specific of all disease-associated mutations in the human genome (Shore et al. 2006; Furuya et al. 2008). *Xin actin-binding repeat containing 2* (*XIRP2*) also known as c*ardiomyopathy-associated protein 3* (*CMYA3*) is also located on HSA2q24.3 and contains 28 Xin repeats which define a novel, repetitive actin-binding motif (Pacholsky et al. 2004). Xin protein is found primarily in the intercalated discs of cardiomyocytes and the myotendinous junctions of skeletal muscle cells, both specialized attachment sites of the myofibrillar ends to the sarcolemma. The Xin repeats define a novel, repetitive actin-binding motif present in at least 2 different muscle proteins. These Xin-repeat proteins therefore constitute the first 2 members of a novel family of actin-binding proteins. Myomaxin, the mouse orthologue of a partial human cDNA of *XIRP2*, is potentially playing a role in regulating cytoarchitectural integrity in striated muscle (Huang et al. 2006). The GALNT13 protein is a member of the uridine diphosphate-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase family, which initiate O-linked glycosylation of mucins (Zhang et al. 2003). Expressed sequence tags of *GALNT13* could be identified in the Equine Articular Cartilage cDNA Library *Equus caballus* (http://www.ncbi.nlm.nih.gov/sites/entrez).

The objective of this study was to confirm and to narrow down this QTL using 71 SNPs of 18 genes in this OC-QTL region on ECA18 at 45.9–87.6 cM (26.15–67.02 Mb on EquCab2.0) and then to employ these intragenic SNPs for association analyses with fetlock OC, fetlock OCD, and hock OC.

Material and Methods

Phenotypic Traits and Sampling of Animals

Radiographic findings were interpreted as OC only in the case when the predilection sites of the fetlock or hock joints were affected by generally accepted signs for this disease (Jeffcott 1991; Wittwer et al. 2006). The predilection site for OC in fetlock joints is the dorsal aspect of the sagittal ridge of the third metacarpal/metatarsal bone. In hock joints, the predilection sites are the intermediate ridge of the distal tibia, the lateral trochlear ridge of the talus, and the lateral/medial malleolus of the tibia (Wittwer et al. 2006). Criteria for OC included the presence of osteochondral fragments, irregular texture of the bone with variable radiopacity, and changes of the regular bone contour such as smoothly flattened, irregularly flattened, smaller, or larger concavity at the mentioned predilection sites. OCD was diagnosed when osteochondral fragments in the joint space were visible at the predilection sites of the fetlock joints and were treated as affected by fetlock OCD in this study. Animals without any signs of radiographic changes of OC at these sites were classified as free from OC and OCD. In addition to OC, we differentiated POFs at the attachment sites of the short sesamoidean ligaments to the proximal phalanx.

For the linkage analysis including 71 SNPs and 19 microsatellites, 4 half-sib families with the highest information content for the QTL on ECA18 were chosen and all available 45 progeny as well as their 35 dams and 4 sires were genotyped. In these 4 half-sib families, there were horses with no signs of OC or OCD and horses affected with fetlock OC (fetlock OCD) and horses with hock OC (Supplementary Table 1). For development of informative SNPs, we included 8 unrelated SGC stallions. The association analysis included 96 horses with an age at radiological examination older than 12 months, belonging to the 9 half-sib families employed in the whole-genome linkage analysis by Wittwer et al. (2007b) (Supplementary Table 2).

Identification of SNPs

Homology between ECA18 and HSA2q has been shown previously (Chowdhary et al. 2003; Penedo et al. 2005; Perrocheau et al. 2006; Swinburne et al. 2006; Wagner et al. 2006) and could be confirmed by an in silico analysis using

60 STS markers and the second horse genome assembly (EquCab2.0). These maps can be used to develop high-density maps of SNPs for fine mapping and association analyses. Recently, a genome scan for OC in horses had shown a QTL on ECA18 (Wittwer et al. 2007b). Based on the comparative map between ECA18 and HSA2q, we chose 20 genes (*ACVR1, ARHGAP15*, *COL3A1, COL5A2, FAP, FRZB, GALNT5, GALNT13, GRB14, ITGA6, LRP2, NAB1, NR4A2, PRPF40A, SLC4A10, SLC40A1, STAM2, TNFAIP6, XIRP2, ZNF804A*), which were evenly spaced along HSA2q from 143.6 to 191.3 Mb (NCBI map viewer, Build 36.2) to cover this region in 1- to 5-Mb intervals (Supplementary Table 3). Whole-genome shotgun sequences and expressed sequence tag sequences (ESTs) were employed to design primer pairs using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). We screened all these 20 genes mapping on ECA18 for SNPs using primarily intronic sequences (Supplementary Table 3). The PCR products were sequenced on a MegaBACE 1000 (GE Healthcare, Freiburg, Germany) automated capillary sequencer, and the resulting sequences were searched for SNPs by visual inspection using Sequencher 4.7 (GeneCodes, Ann Arbor, MI). The sequencing reactions were carried out using the DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare). With exception of *COL5A2* and *SLC4A10*, we could find polymorphic SNPs in these genes.

Data Analysis

Multipoint nonparametric linkage analysis was performed using Merlin, version 1.1.1 (multipoint engine for rapid likelihood inference, Center for Statistical Genetics, University of Michigan, MI; Abecasis et al. 2002). *Z*means and logarithm of the odds scores were used to test for the proportion of alleles shared by affected individuals identical by descent. All markers used were tested for correctness of Mendelian inheritance using PEDSTATS (Wigginton and Abecasis 2005).

SNPs located within the significantly linked region (at a chromosome-wide $P < 0.05$) with the OC condition in the genotyped families were further evaluated in an association analysis. The ALLELE procedure of the software package SAS/Genetics (Statistical Analysis System, version 9.1.3, SAS Institute Inc., Cary, NC, 2007) was used to estimate the observed heterozygosity (H_O), the polymorphism information content and to test for Hardy–Weinberg equilibrium. Association was tested for all 71 SNPs of the 18 genes in preliminary analyses using the CASECONTROL procedure and χ^2 tests of SAS/Genetics, version 9.2, in order to test for linkage disequilibrium between the different OC phenotypes and marker genotypes, marker alleles, and number of alleles. In order to control for systematic environmental, the family structure of our data and quantitative genetic effects for SNPs exhibiting significant χ^2 tests in the CASECONTROL procedure, we employed an animal model for the association analysis using the genotypes of these SNPs and phenotypes for fetlock OC, fetlock OCD, and hock OC. The linear animal model included the fixed effects of the particular genotype, sex (male with 19 and females with 77 horses), month of birth (month, with 3 levels, January to March, April, May to July, with 43, 34, and 19 horses), age at radiological examination of the horse (age, with 2 levels, 11–17 months and >17 months, with 50 and 46 horses), interaction between age at radiological examination and sex, interaction between month of birth and sex, and the random additive genetic effect of the animal (a) including 966 horses of the pedigree.

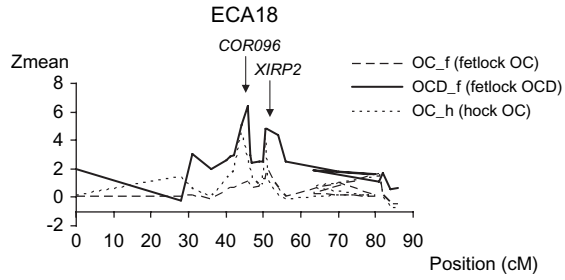

Figure 1. *Z*means for the QTL on ECA18 for SGC horses using 19 microsatellites and 71 SNPs in 18 positional candidate genes.

Table 1. Number of controls and affected horses, MAF, and significant results of the association analysis using χ^2 tests for 2 SNPs located in the positional candidate gene *XIRP2* on ECA18 for fetlock OC, fetlock OCD, and hock OC

Trait/SNP	Control horses *n*–MAF	Affected horses *n*–MAF	χ^2 tests for association			Error probability		
			Genotype	Allele	Trend	Genotype	Allele	Trend
Fetlock OC								
AJ885515:g.159A>G	10–0.10	68–0.42	8.02	7.55	7.21	0.018	0.006	0.007
AJ885515:g.445T>C	10–0.11	68–0.42	6.39	6.28	5.89	0.041	0.012	0.015
Fetlock OCD								
AJ885515:g.159A>G	10–0.10	29–0.48	10.10	9.21	8.98	0.006	0.002	0.003
AJ885515:g.445T>C	10–0.11	29–0.48	8.40	7.87	7.60	0.015	0.005	0.006
Hock OC								
AJ885515:g.159A>G	10–0.10	58–0.41	6.47	6.89	6.05	0.039	0.009	0.014
AJ885515:g.445T>C	10–0.11	58–0.40	5.06	5.68	4.86	0.080	0.017	0.027

MAF, minor allele frequency.

$$y_{ijklmn} = \mu + \text{genotype}_i + \text{sex}_j + \text{month}_k + \text{age}_l + \text{age} \times \text{sex}_{jl} + \text{month} \times \text{sex}_{jk} + a_m + e_{ijklmn}.$$

Estimates of the quantitative additive genetic and residual effects were taken from Wittwer et al. (2007a). Heritabilities for hock OC, fetlock OC, fetlock OCD, and fetlock/hock OC of SGC horses were 0.04, 0.08, 0.16, and 0.17, respectively. The additive genetic effects of the SNPs were estimated by pairwise comparisons of the half of the least square means between the 2 homozygous genotypes, and the dominance effect was calculated as the deviation of the least square means of the heterozygotes from the average of the 2 homozygous genotypes. Significance was tested using *F* tests. The analyses were performed using PEST (Groeneveld 1990).

Results and Discussion

A QTL-containing region at 45.9–87.6 cM on ECA18 was refined to an interval of 31.0–56.1 cM using a total of 19 microsatellites and 71 SNPs in 18 positional candidate genes for a multipoint nonparametric linkage analysis in 4 informative paternal half-sib families. A high-resolution comparative map of ECA18 was used to locate these positional candidate genes and microsatellite markers within this QTL region. In addition, the genomic location of the candidate genes was confirmed on the horse genome assembly EquCab2.0. For fetlock OCD, the highest *Z*mean of 6.37 (genome-wide $P < 0.001$) was reached between 44.0 and 45.9 cM at a *GALNT13* intragenic SNP and the microsatellite *COR096* (Figure 1). The *Z*mean for hock OC peaked at 44.0 cM (*GALNT13*) and at 50.5 cM (*XIRP2*) with values of 4.56 and 4.23 for *Z*means (genome-wide $P < 0.001$). The SNPs of the *ACVR1* gene were located at about 47.5 cM, and their *Z*means reached values of nearly similar size for fetlock OCD and hock OC.

A total of 23 SNPs in the candidate genes *GALNT13* (3 SNPs), *ACVR1* (14 SNPs), and *XIRP2* (6 SNPs) was further used for association analysis with fetlock OC, fetlock OCD, and hock OC. Two SNPs in *XIRP2* (AJ885515:g.159A>G, AJ885515:g.445T>C in intron 2) were significantly associated ($P = 0.002$–0.05) with fetlock OC, fetlock OCD, and hock OC (Table 1). The candidate gene *XIRP2* (*CMYA3*) is located in the peak region of the QTL (Figure 1). The SNPs in the *GALNT13* and *ACVR1* genes were not consistently associated with OC or OCD in fetlock or hock joints (Supplementary Table 4), and in models regarding stratification of data, these SNPs did not reveal significant associations ($P > 0.10$). In addition, we could demonstrate that the SNPs in the remaining 15 genes did not show an association with any of the OC conditions investigated here (data not shown), and this may further corroborate the possible involvement of *XIRP2* in equine OC.

Table 2. Additive genetic effects for associated SNPs in the *XIRP2* gene estimated in a linear animal model for fetlock OC, fetlock OCD, and hock OC and their corresponding error probabilities (*P*)

Trait/SNP	Additive genetic effect ± standard error	Error probability (*P*)
Fetlock OC		
AJ885515:g.159A>G	0.150 ± 0.054	0.007
AJ885515:g.445T>C	0.145 ± 0.055	0.010
Fetlock OCD		
AJ885515:g.159A>G	0.270 ± 0.092	0.006
AJ885515:g.445T>C	0.266 ± 0.095	0.009
Hock OC		
AJ885515:g.159A>G	0.155 ± 0.062	0.014
AJ885515:g.445T>C	0.151 ± 0.063	0.019

Table 3. Genotypic risks for developing fetlock OC, fetlock OCD, or hock OC associated with the intragenic SNP (AJ885515:g.159A>G) of *XIRP2* estimated in a linear animal model

Genotypes compared	Contrasts between genotypes (Δ ± standard error)	*P*	Relative risk to develop OC or OCD for the susceptible genotype A/A or A/G
Fetlock OC			
A/A–A/G	0.060 ± 0.106	0.579	1.07
A/A–G/G	0.300 ± 0.108	0.007	1.48
A/G–G/G	0.240 ± 0.081	0.004	1.38
Fetlock OCD			
A/A–A/G	0.110 ± 0.183	0.554	1.13
A/A–G/G	0.539 ± 0.184	0.006	2.38
A/G–G/G	0.430 ± 0.139	0.004	2.10
Hock OC			
A/A–A/G	<0.001 ± 0.126	0.997	1.00
A/A–G/G	0.310 ± 0.123	0.014	1.49
A/G–G/G	0.310 ± 0.098	0.003	1.49

Table 4. Genotypic risks for developing fetlock OC, fetlock OCD, or hock OC associated with the intragenic SNP (AJ885515:g.445T>C) of *XIRP2* estimated in a linear animal model

Genotypes compared	Contrasts between genotypes (Δ ± standard error	*P*	Relative risk to develop OC or OCD for the susceptible genotype T/T or T/C
Fetlock OC			
T/T–T/C	0.070 ± 0.109	0.498	1.09
T/T–C/C	0.290 ± 0.109	0.010	1.44
T/C–C/C	0.216 ± 0.083	0.011	1.33
Fetlock OCD			
T/T–T/C	0.116 ± 0.189	0.545	1.14
T/T–C/C	0.532 ± 0.190	0.009	2.32
T/C–C/C	0.417 ± 0.145	0.008	2.03
Hock OC			
T/T–T/C	0.025 ± 0.131	0.852	1.03
T/T–C/C	0.302 ± 0.125	0.019	1.45
T/C–C/C	0.278 ± 0.103	0.010	1.42

The *XIRP2* gene-associated SNPs (AJ885515:g.159A>G, AJ885515:g.445T>C) were tested in more refined statistical models (Table 2). Both intronic SNP genotypes in *XIRP2* were significantly associated with fetlock OC ($P = 0.004$–0.016), fetlock OCD ($P = 0.0085$–0.036), and hock OC ($P = 0.0033$–0.014). The 2 SNPs in intron 2 of *XIRP2* showed significant additive genetic effects for fetlock OC, fetlock OCD, and hock OC in the size of 0.15–0.27 ($P = 0.01$–0.02) and for all OC conditions nonsignificant dominance effects. The alleles of these 2 SNPs were in complete linkage disequilibrium, and thus, one SNP is sufficient to explain this association with fetlock and hock OC. Thus, we assume that mutations in *XIRP2* may confer susceptibility to the development of fetlock and hock OC in SGC horses.

All horses homozygous for the susceptible genotypes of the *XIRP2* SNPs (A/A and T/T) were affected by fetlock OC ($n = 12$), fetlock OCD ($n = 6$), and hock OC ($n = 11$). Employing linear animal model estimates, affection risks of these homozygous genotypes were at 93–94% for fetlock OC, at 89–92% for fetlock OCD, and at 96% for hock OC. All contrasts between the 2 homozygous genotypes were significant (Tables 3 and 4). Only 2 horses heterozygous for the *XIRP2* SNPs 159A>G and 445T>C were not affected by any signs of OC in fetlock or hock joints, whereas all other heterozygous horses showed signs of OC. Heterozygous horses were at similarly high risk levels for OC like the susceptible homozygous genotypes. The relative risks for OC associated with the homozygous and heterozygous susceptible genotypes were at 1.33–2.38. All dominance effects for all SNPs in the 3 candidate genes were nonsignificant in the animal model (data not shown).

Our results indicate that *XIRP2* is a strong candidate responsible for OC and OCD in fetlock and hock joints of SGC horses. *XIRP2* was found highly significantly upregulated in myocardial tissue of mice with angiotensin II (Ang II)-induced hypertensive/ischemic cardiomyopathy. Its upregulation was due to Ang II-induced myocardial damage (Duka et al. 2006). Mutation and expression analyses of the associated gene *XIRP2* will be helpful to clarify which role this gene plays in the etiopathogenesis of the OC syndrome in horses. The present analysis is a step toward unravelling genes responsible for equine OC and is a further report on gene-associated markers in population-wide linkage disequilibrium with equine OC and OCD in fetlock and hock joints (Wittwer et al. 2008).

Supplementary Material

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

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Corresponding Editor: Ernest Bailey