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Candidate gene analysis of osteochondrosis in Spanish Purebred horses

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Summary

Equine osteochondrosis (OC) is a frequent developmental orthopaedic disease with high economic impact on the equine industry and may lead to premature retirement of the animal as a result of chronic pain and lameness. The genetic background of OC includes different genes affecting several locations; however, these genetic associations have been tested in only one or few populations, lacking the validation in others. The aim of this study was to identify the genetic determinants of OC in the Spanish Purebred horse breed. For that purpose, we used a candidate gene approach to study the association between loci previously implicated in the onset and development of OC in other breeds and different OC locations using radiographic data from 144 individuals belonging to the Spanish Purebred horse breed. Of the 48 polymorphisms analysed, three single nucleotide polymorphisms (SNPs) located in the *FAF1*, *FCN3* and *COL1A2* genes were found to be associated with different locations of OC lesions. These data contribute insights into the complex gene networks underlying the multifactorial disease OC, and the associated SNPs could be used in a marker-assisted selection strategy to improve horse health, welfare and competitive lifespan.

Keywords association, *COL1A2*, developmental orthopaedic disease, *Equus caballus*, *FAF1*, *FCN3*, single nucleotide polymorphism

Introduction

The Spanish Purebred horse, or Andalusian horse – the most ancient horse in the Iberian Peninsula (Aparicio 1944) – has been bred mainly for classical dressage since the 15th century (Lenoir 1998), when the Carthusian strain was formed. This is the only strain recognised by the Spanish Purebred studbook, although without genetic support (Valera *et al.* 2005). The Spanish Purebred horse, apart from being the most important equine breed in Spain where it is also used for other sport activities and recreation, was involved in the formation of breeds such as Lippizan, Lusitano and native horse American strains.

Osteochondrosis (OC) is a developmental orthopaedic disease frequent in horses that can be defined as a local alteration in endochondral ossification, characterised by abnormal chondrocyte differentiation and maturation in young animals (Rejnö & Strömberg 1978). The clinical manifestations of OC include abnormalities in the ossification process that lead to locally thickened cartilage plugs and flaps, the formation of necrotic areas, synovial effusions and eventually the formation of loose fragments known as osteochondritis dissecans (OCD) (Brama 2009). The locations most commonly affected are the fetlock, hock and stifle joints, usually showing a bilateral and symmetrical pattern (McIlwraith 2005). The multifactorial origin behind the aetiology of OC is not completely understood. The onset of clinical symptoms associated with the start of training practices points towards biomechanical influences (Jeffcott 1997; Wittwer *et al.* 2006); however, alterations in the vascular supply during epiphyseal growth seem also important in the onset of OC (Olstad *et al.* 2011). There is also a well-documented genetic component with heritability estimations in different horse breeds in the range of $h^2 = 0.10–0.52$ (Grondahl & Dolvik 1993; Stock *et al.* 2005; Wittwer *et al.* 2007; Van Grevenhof *et al.* 2009), and the implication of other environmental factors such as diet, increased growth or reduced or irregular physical activity has also been described (Lepeule *et al.* 2013). All these variables complicate the management of this disease that may lead to premature retirement of the animal as a result of chronic pain and lameness (Stock & Distl 2006).

Microsatellite-based whole-genome scans (e.g. Dierks *et al.* 2007; Wittwer *et al.* 2008) and genome-wide

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association studies (GWAS) (e.g. Dierks *et al.* 2010; Lykkjen *et al.* 2010; Corbin *et al.* 2012; Teyssèdre *et al.* 2012) have identified different putative quantitative trait loci (QTL) and single nucleotide polymorphisms (SNPs) associated with the development of OC lesions in Dutch Warmblood, Thoroughbred, Hanoverian Warmblood, French Trotters, Standardbred, Norwegian Standardbred and South German Coldblood breeds. These results have shown that the genetic background of OC may include different genes affecting several locations, and there is no clear evidence of replication of the loci identified in other breeds, such as the Spanish Purebred horse. In this study, we performed an association study between loci previously implicated in OC development in other breeds by genotyping either associated SNPs or SNPs located in candidate genes (Table 1) and different OC locations, using radiographic data from 144 Spanish Purebred horses to identify the genetic determinants in this breed.

Materials and methods

Animals and phenotypic data

Radiographic data were collected from 144 yearling Spanish Purebred horses born between 2000 and 2012 and registered in the studbook of the breed in Spain to assess the prevalence of the various radiographic manifestations of OC in a cross-sectional study. To reduce the environmental effects, all animals belonged to one of the most important Spanish Purebred horse stud, an open stud that every year adds new stallions to the herd, offering a wide representation of the breed that included the offspring of 44 sires and 116 dams. Animals were sedated with a combination of 0.01–0.02 mg/kg IV of detomidine hydrochloride (Domidine, Divasa-Farmavic) and 0.02–0.03 mg/kg IV of butorphanol tartrate (Torbugesic, Fort Dodge Veterinaria) and then scored for OC based on direct radiographs (Tru Dr LX) of the left and right femoropatellar (FP), metacarpophalangeal (MCP), metatarsophalangeal (MTP) and tarsocrural (TC) joints. A total of 10 radiographic projections were analysed, including lateromedial views of the MCP and MTP joints, dorsolateral plantomedial oblique and dorsomedial plantolateral oblique views of the TC joint as well as the lateromedial view of the FP joint. In each animal, 28 predilection sites were scrutinised for the presence of OC following Van Grevenhof *et al.*'s (2009) guidelines. At each site, OC was scored on a categorical scale from 0 to 4 (Table 2) (Dik *et al.* 1999). Score 0 indicates normal joint contour, scores 1 and 2 indicate flattened bone contours, and scores 3 and 4 indicate the presence of fragments. The same two equine veterinarians took all radiographs and judged the grade of the lesions in all the animals included in the study.

Prevalence was calculated at the level of joint (MCP, MTP, TC, FP), symmetry and gender. Irregular bone surface, concavities or osseous fragments located at these predilection sites were interpreted as signs of OC (Van Grevenhof *et al.* 2009). Horses with pathological changes in these joints other than OC were not included in the study. Animals without any signs of radiographic changes for all 28 predilection sites examined in the MCP, MTP, TC and FP joints were classified as free from OC and defined as controls ($n = 74$). The phenotypic traits analysed included: (i) grade of OC, defined as the global categorical score in a scale from 0 to 4 assigned by the veterinarians taking into account all the OC manifestations present in an individual; (ii) the presence of OC lesions in the MCP, MTP, TC and FP joints; and (iii) total, defined as the arithmetic sum of the scores of all lesions in different locations per individual, where 0 represented individuals without lesions, 1 represented individuals for which the sum of lesions was between 1 and 2, 2 represented individuals for which the sum of lesions was between 3 and 4, 3 represented individuals for which the sum of lesions was between 5 and 7 and 4 represented individuals for which the sum of lesions was between 8 and 13.

SNP selection and genotyping

A total of 48 SNPs were selected from the literature or the GenBank database (http://www.ncbi.nlm.nih.gov) or were located by sequencing an initial reference panel consisting of five samples from Spanish Purebred horses diagnosed with OC and five controls. The list of candidate genes and markers associated with different types of OC lesions and different locations in several equine breeds included in this study are provided in Table 1. Additionally, we also included nine candidate genes described in porcine and humans as implicated in the onset of OC.

The 48 selected SNPs were included in three different Multiplex-Capillary Primer Extension assays following the procedure described in Sevane *et al.* (2010). The multiplex and primer extension primers and PCR conditions are shown in Table S1. Replication of SNP genotyping was performed in 5% of the samples for repeatability purposes, and Mendelian inheritance was checked in four trios for reliability.

Statistical analysis

Phenotypic data, including grade, left MCP, left MTP, left TC, left FP, right MCP, right MTP, right TC, right FP and total traits, had to be transformed to comply with normality conditions underlying the linear model by $\log(1 + Y)$ transformation. SNPs with minor allele frequency (MAF) less than 0.05 were excluded from the association analysis to avoid bias of the data. Linear regression analysis was then applied to test the associations between genotypes and phenotypes using R programming (http://www.r-project.org) and the LME4 statistical package, which fits linear models and generalised linear mixed models (GLMMs) to

Table 1 Candidate genes and markers previously associated with the presence of osteochondrosis lesions in several equine breeds, pigs and humans.

Locus[1]	Associated marker[2]	Breed/species	References	dbSNP for new candidate markers
Chr3-808543	rs68603064	Dutch Warmblood	Orr *et al.* (2012)	–
GPR63	rs68934440			–
MMP13	–	–	Riddick *et al.* (2012) & Mirams *et al.* (2009)	rs68699208
PDGFA	–	–	Riddick *et al.* (2012)	rs68912248
MMP3	–	–		ss1868861729
PTHLH	–	–		rs68648021
KLF3	rs68512502	Thoroughbred	Corbin *et al.* (2012)	–
Chr4-859811	rs69589048	Thoroughbred, Hanoverian Warmblood	Corbin *et al.* (2012) & Komm (2010)	–
Chr18-410967	rs69134570	Thoroughbred, Hanoverian Warmblood	Corbin *et al.* (2012) & Lampe *et al.* (2009a)	–
STAG3	rs68913180	French Trotters	Teyssèdre *et al.* (2012)	–
CRISP3	rs68914778			–
FAM184B	rs68534880			–
USP31	rs68599858			–
MCPT1	rs68945241			–
ANKRD32	rs68945244			–
Chr15-320636	rs69009627			–
Chr15-320532	rs69008423			–
TLK2	–	Standardbred	Austbø *et al.* (2010)	rs68930565
C16orf72	–			rs68938090
NCDN	g.1758T>C AAWR02028119	Hanoverian Warmblood	Dierks *et al.* (2010)	–
FCN3	g.8256C>T AAWR02028318			–
MECR	g.30801G>T AAWR02028280			–
HECW1	g.79876T>C AAWR02031763	Hanoverian Warmblood	Komm (2010)	–
Chr4-893170	rs69473630			–
Chr4-851132	rs69609158			–
SGK1	rs68861801	Norwegian Standardbred	Lykkjen *et al.* (2010)	–
	rs68863106			–
PTH2R	–	Hanoverian Warmblood	Lampe *et al.* (2009a)	ss1868861726
COL24A1	–	Hanoverian Warmblood	Lampe *et al.* (2009b)	rs69511701
DOCK3	g.19744C>T AAWR02008465	Hanoverian Warmblood	Lampe *et al.* (2009c)	–
PRKCD	–			rs69109850
COL1A1	–	–	Mirams *et al.* (2009)	rs68845626
COL1A2	–	–		rs69595660
COL10A1	–	–		ss1868861722
RUNX2	–	–		ss1868861723
XIRP2	g.159A>G AJ885515	South German Coldblood	Wittwer *et al.* (2009)	–
AOAH	g.703A>G AJ543065	South German Coldblood	Wittwer *et al.* (2008)	–
FAF1	–	Porcine	Rangkasenee *et al.* (2013a)	rs68596812
PTH1R	–			rs69065715
TBX5	–	Porcine	Rangkasenee *et al.* (2013b)	ss1868861736
TGFB1	–	Porcine	Laenoi *et al.* (2012)	rs68989779
MGP	–	Porcine	Laenoi *et al.* (2010)	rs68649284
BAK1	–	Humans	Wang *et al.* (2012)	ss1868861738
APAF1	–			ss1868861732
	–			ss1868861733
CASP6	–			rs68604989
IGFBP2	–			rs68664157
COL5A2	–			rs69137084

[1]Locus symbol or single nucleotide polymorphism (SNP) number.
[2]GenBank accession numbers for *Equus caballus* sequences including the interrogated SNPs or dbSNP accession numbers.

Table 2 Prevalence of osteochondrosis lesions based on the radiographic data of 144 Spanish Purebred horses according to Dik *et al.*'s (1999) classification.[1]

	Normal 0	Minimal 1	Mild 2	Moderate 3	Severe 4
Grade	74	21	19	18	12
Left MCP	130	9	4	1	–
Left MTP	125	9	5	5	–
Left TC	110	16	2	8	8
Left FP	141	2	–	–	1
Right MCP	133	8	2	1	–
Right MTP	118	12	7	6	–
Right TC	105	16	3	15	4
Right FP	141	–	2	–	1
Total[2]	74	22	25	17	6

MCP, metacarpophalangeal; MTP, metatarsophalangeal; TC, tarsocrural; FP, femoropatellar.
[1]0, rounded bone contour, diffuse density of the subchondral bone, fragments absent; 1, smoothly flattened bone contour, obscure lucency of the subchondral bone, fragments absent; 2, irregularly flattened bone contour, obvious ill-bordered local lucency of the subchondral bone, fragments absent; 3, small, rounded/irregular concavity in the bone contour, obvious well-defined local lucency of the subchondral bone, fragments <5 mm; 4, large, rounded/irregular concavity in the bone contour, obvious well-defined extensive lucency of the subchondral bone, fragments ≥5 mm.
[2]Arithmetic sum of the scores of all lesions in different locations per individual. 0, individuals without lesions; 1, sum of lesions 1 and 2; 2, sum of lesions 3 and 4; 3, sum of lesions 5 to 7; 4, sum of lesions 8 to 13.

data (Bates & Maechler 2008). The main assumption was that the SNP effect on any of the traits is additive.

The effect of the SNP on each of the traits was estimated by including them as a covariate into a linear model. The model used in this study was as follows:

$$Y_{ij} = BY_i + \alpha G_{ij} + e_{ij},$$

where Y_{ij} is the trait registered in the jth individual for the ith birth year, BY_i is the fixed effect of the ith birth year, α is the regression coefficient for the relation between Y and G, G_{ij} is the ordered genotype constants with values 1, 2 or 3, e_{ij} are independent $N(0, \sigma^2)$ and $j = 1,\ldots\ldots,144$.

In order to correct for multiple testing in each group, a permutation analysis was carried out to calculate the experiment-wise significance threshold within each trait (Churchill & Doerge 1994). For each permutation, SNP genotypes were randomised across all animals. The effect of each SNP was then estimated, and the maximum F statistic across all SNPs was used to calculate the distribution of the null hypothesis. A total of 10 000 permutations were used to calculate the null distribution from which the 5% experiment-wise significance thresholds were inferred.

Fisher's exact test for Hardy–Weinberg equilibrium across loci was performed with GENEPOP 1.2 (Raymond & Rousset 1995). Spearman's correlations were determined between the different location of lesions using the CORR procedure implemented in the SAS statistical package v. 9.1.3 (SAS Institute, Inc. 2009) and considering the whole set of data on all animals.

Results

The 144 Spanish Purebred horses included in the study were radiographed and classified according to the severity and location of the lesions in the fetlock, hock and stifle joints, symmetry and gender. Of this sample, 52.8% were male and 47.2% were female. In concordance with previous studies, no gender influence has been detected in the development of OC ($P = 0.434$) (Sandgren *et al.* 1993; Van Weeren & Barneveld 1999; Douglas 2003; Hernández 2003; Boado & López-Sanromán 2015). The radiographic assessment was performed when foals were 12 months old following the classification of Dik *et al.* (1999). In this classification, score 0 indicates normal joint contour (51.4% of the animals), scores 1 and 2 indicate flattened bone contours (27.8%) and scores 3 and 4 indicate the presence of fragments (20.8%). The location and the degree of lesions in the 144 individuals analysed are shown in Table 2.

The most frequent location of OC lesions was the hock (TC) joint, with a slightly higher prevalence in the present study (23.6% and 26.6% in the left and right hock respectively), compared with previous data that showed a prevalence of 19% in the Spanish Purebred horse (Novales *et al.* 1999; Hernández 2003). MTP fetlocks displayed a higher prevalence (15.3%) of OC manifestations than did MCP predilection sites (8.7%), which is in agreement with the results reported by Hernández *et al.* (2006). The prevalence in the stifle (FP) was low (2.1%), also in concordance with previous publications (Novales *et al.* 2000). Spearman's correlations were significant ($P < 0.001$) and positive between the prevalence of lesions in symmetric joints (Table 3), as

Table 3 Correlations between the prevalence of lesions in the fetlocks (MCP, MTP), hocks (TC) and stifles (FP) joints in the Spanish Purebred horse.

	Left MCP	Left MTP	Left TC	Left FP	Right MCP	Right MTP	Right TC
Left MTP	0.56*						
Left TC	0.03	−0.02					
Left FP	0.11	0.08	−0.08				
Right MCP	0.51*	0.49*	0.09	−0.04			
Right MTP	0.40*	0.49*	0.04	0.08	0.41*		
Right TC	0.07	0.12	0.53*	0.13	0.09	0.09	
Right FP	−0.05	−0.06	0.02	0.31*	−0.04	−0.07	0.13

MCP, metacarpophalangeal; MTP, metatarsophalangeal; TC, tarsocrural; FP, femoropatellar.
*$P < 0.001$.

Table 4 Forty-eight polymorphisms genotyped in 144 Spanish Purebred horses, dbSNP accession number or location, allele frequencies per osteochondrosis (OC) grade (Dik *et al*. 1999) and genotype frequencies.

			Frequency of allele 1								
			OC grade						Genotype frequency		
Locus[1]	GenBank/dbSNP[2]	Allele 1/Allele 2	0	1	2	3	4	Overall	11	12	22
Chr3-808543	rs68603064	T/C	0.51	0.60	0.45	0.53	0.42	0.51	0.222	0.576	0.201
Chr18-410967	rs69134570	T/C	0.29	0.19	0.26	0.28	0.33	0.27	0.069	0.410	0.521
USP31	rs68599858	A/G	0.55	0.45	0.58	0.50	0.67	0.54	0.271	0.542	0.188
Chr4-859811	rs69589048	T/C	0.56	0.57	0.61	0.42	0.54	0.55	0.278	0.542	0.181
Chr4-851132[3]	rs69609158	T/C	0.03	0.07	0	0	0	0.03	0	0.056	0.944
FAM184B	rs68534880	A/G	0.06	0.07	0	0.11	0.04	0.06	0	0.118	0.882
FAF1	rs68596812	C/G	0.95	0.90	0.92	0.92	0.92	0.93	0.867	0.126	0.007
ANKRD32	rs68945244	A/G	0.28	0.43	0.24	0.28	0.33	0.30	0.049	0.507	0.444
CRISP3	rs68914778	G/C	0.55	0.57	0.53	0.50	0.46	0.53	0.250	0.569	0.181
KLF3	rs68512502	A/G	0.42	0.17	0.34	0.44	0.50	0.38	0.153	0.458	0.389
SGK1	rs68861801	T/C	0.07	0	0.05	0.03	0.04	0.05	0.007	0.083	0.910
	rs68863106	T/C	0.07	0	0.05	0.03	0.04	0.05	0.014	0.076	0.910
NCDN	g.1758 T>C AAWR02028119	T/C	0.80	0.81	0.68	0.89	0.88	0.81	0.667	0.278	0.056
DOCK3	g.19744 C>T AAWR02008465	T/C	0.24	0.24	0.24	0.22	0.29	0.24	0.056	0.375	0.569
XIRP2	g.159 A>G AJ885515	T/C	0.28	0.24	0.39	0.33	0.42	0.31	0.083	0.451	0.465
AOAH[3]	g.703 A>G AJ543065	A/G	1	0.98	1	1	1	0.997	0.993	0.007	0
GPR63	rs68934440	A/C	0.83	0.93	0.71	0.78	0.79	0.82	0.674	0.292	0.035
STAG3	rs68913180	A/G	0.34	0.45	0.24	0.28	0.13	0.32	0.186	0.264	0.550
MMP3	ss1868861729	T/A	0.09	0.05	0	0.03	0	0.06	0	0.112	0.888
APAF1	ss1868861732	A/G	0.12	0.10	0.11	0.17	0.08	0.12	0	0.238	0.762
	ss1868861733	T/C	0.14	0.14	0.13	0.11	0.17	0.14	0.021	0.229	0.750
C16orf72	rs68938090	A/G	0.11	0.07	0.18	0.11	0.25	0.13	0.007	0.243	0.750
TLK2	rs68930565	A/G	0.58	0.36	0.63	0.53	0.54	0.55	0.264	0.563	0.174
PRKCD	rs69109850	A/G	0.06	0.02	0.05	0.06	0.04	0.05	0	0.104	0.896
FCN3	g.8256 C>T AAWR02028318	T/C	0.04	0.14	0.05	0.11	0.17	0.08	0.007	0.140	0.853
PTH2R	ss1868861726	T/A	0.88	0.86	0.87	0.85	0.83	0.87	0.746	0.239	0.014
MGP	rs68649284	T/G	0.40	0.50	0.37	0.33	0.50	0.41	0.160	0.500	0.340
RUNX2	ss1868861723	C/G	0.84	0.88	0.87	0.86	0.83	0.85	0.729	0.250	0.021
Chr15-320636	rs69009627	A/G	0.56	0.69	0.53	0.61	0.58	0.58	0.333	0.500	0.167
COL24A1	rs69511701	T/C	0.45	0.33	0.39	0.28	0.38	0.40	0.174	0.451	0.375
Chr15-320532	rs69008423	A/G	0.79	0.71	0.84	0.86	0.92	0.80	0.643	0.322	0.035
PTH1R	rs69065715	A/G	0.61	0.64	0.61	0.61	0.71	0.62	0.361	0.521	0.118
COL10A1	ss1868861722	T/C	0.35	0.31	0.42	0.28	0.46	0.35	0.084	0.538	0.378
TBX5	ss1868861736	T/C	0.60	0.60	0.71	0.53	0.54	0.60	0.347	0.507	0.146
Chr4-893170	rs69473630	T/C	0.95	0.95	0.94	0.92	0.96	0.94	0.887	0.113	0
PTHLH	rs68648021	T/C	0.25	0.10	0.21	0.35	0.18	0.23	0.122	0.216	0.662
IGFBP2	rs68664157	T/G	0.37	0.43	0.47	0.39	0.46	0.40	0.167	0.472	0.361
MECR	g.30801 G>T AAWR02028280	T/G	0.09	0.07	0.11	0.11	0.13	0.10	0.014	0.167	0.819
PDGFA	rs68912248	A/G	0.50	0.55	0.53	0.39	0.46	0.49	0.257	0.472	0.271
CASP6	rs68604989	T/C	0.70	0.74	0.74	0.89	0.50	0.72	0.514	0.410	0.076
HECW1	g.79876 T>C AAWR02031763	T/C	0.51	0.43	0.58	0.69	0.58	0.53	0.299	0.472	0.229
COL5A2	rs69137084	T/C	0.09	0.10	0.13	0.17	0.21	0.12	0.021	0.194	0.785
MMP13	rs68699208	T/C	0.16	0.14	0.11	0.08	0.04	0.13	0	0.257	0.743
MCPT1	rs68945241	A/G	0.65	0.76	0.61	0.81	0.79	0.69	0.486	0.410	0.104
TGFB1	rs68989779	A/G	0.22	0.24	0.26	0.22	0.13	0.22	0.049	0.347	0.604
COL1A1	rs68845626	C/G	0.94	0.90	0.89	0.94	1	0.93	0.882	0.104	0.014
BAK1	ss1868861738	A/C	0.48	0.31	0.45	0.36	0.54	0.44	0.264	0.354	0.382
COL1A2	rs69595660	T/C	0.43	0.40	0.53	0.53	0.67	0.47	0.222	0.493	0.285

[1]Locus symbol or single nucleotide polymorphism (SNP) number.
[2]GenBank accession numbers for *Equus caballus* sequences including the interrogated SNPs or dbSNP accession numbers.
[3]SNP with minor allele frequency (MAF) less than 0.05 excluded from the association analysis.

reported by McIlwraith (2005). MCP and MTP fetlocks, both left and right, showed a mean correlation of 0.48, hocks of 0.53 and stifles of 0.31 ($P < 0.001$). As expected, there were no correlations between the prevalence of OC in different anatomical locations of the same animal.

Forty-eight SNPs, some previously associated with different manifestations of OC in several horse breed and others

located in candidate genes from equine, porcine and human studies, were genotyped in the full set of samples. Frequencies of the analysed SNPs per OC grade are shown in Table 4. After eliminating two SNPs with MAF under 0.05 (Chr4:851132 and the SNP in gene *AOAH*, Table 4), 46 polymorphisms were analysed, and three SNPs located in the *FAF1*, *FCN3* and *COL1A2* genes were found to be associated with different locations of OC lesions through linear regression analysis with effects ranging from 0.30 to 0.69 standard deviation units (Table 5). Significant and suggestive (F Reg > 8) associations are shown. The GG genotype of the intronic SNP rs68596812 located in the *FAF1* gene was significantly associated with an increase in the prevalence of OC in the right MTP fetlock of 42.7% when compared with the CC genotype. The frequencies of the GG, GC and CC genotypes in the population were 0.01, 0.13 and 0.86 respectively. The TT genotype of the *FCN3* intronic SNP g.8256C>T accounted for an increase in the prevalence of OC in the right stifle of 20.1% when compared with the CC genotype (TT genotype frequency, 0.01; TC, 0.14; CC, 0.85). And the genotype TT of the *COL1A2* intronic SNP rs68845626 caused an increase in the sum of OC lesions in different locations in the same animal of 30.2% when compared with the CC genotype (TT genotype frequency 0.22; TC, 0.50; CC, 0.28).

Discussion

In this candidate gene approach, three SNPs in the *FAF1*, *FCN3* and *COL1A2* genes were found to be associated with different locations of OC lesions in the Spanish Purebred horse. Apart from the *FCN3* gene, which is included in a previously identified QTL, both the *FAF1* and *COL1A2* genes are not included in any reported OC QTL (see Table 1 for references). Moreover, among the 24 SNPs previously associated with OC lesions in other equine breeds, only the *FCN3* SNP association was validated in the Spanish Purebred horse. The reasons for this low validation success may be (Corbin *et al.* 2012): (i) the effects of the QTL detected using primarily linkage-based analyses are usually large, but their locations are imprecise and (ii) although GWAS overcome some of the limitations of these analyses, few putative correspondences between QTL have been described so far, which may be due to factors such as false positives, differences in the significance thresholds used, subsequent studies underpowered to detect them, differing phenotype definitions, small sample sizes or breed differences. The three associated markers were in Hardy–Weinberg equilibrium, which may be due to the short time since any selection pressure against OC has been performed in this breed (~10 years). Taking into account the average generation interval for the Spanish Purebred horse (~10.1 years) (Valera *et al.* 2005), approximately only one generation has been under selection for this trait. Moreover, OC is not a trait systematically registered, lacking accurate data on the genetic merit for the quality of each joint and selecting only against OC. All these factors can explain the lack of changes in SNP allelic frequencies observed in this study.

The *Fas (TNFRSF6) associated factor 1* gene (*FAF1*) has been previously associated with OC lesion scores of all joints inspected in pigs, including the humerus, condylus medialis humeri, condyles lateralis humeri, radius and ulna proximal, head of femur, condylus medialis femoris and distal epiphyseal cartilage of the ulna (Rangkasenee *et al.* 2013a). The Fas antigen is a transmembrane receptor that is known to mediate apoptosis in different tumour and hematopoietic cells (Chu *et al.* 1995). The protein coded by the *FAF1* gene can initiate or enhance the apoptotic cascade initiated by the Fas antigen. This protein also acts as a negative modulator of osteoblast differentiation induced by Wnt, a highly conserved signal protein family that regulates cell proliferation and differentiation and, hence, is implicated in the control of numerous biological processes including embryonic development and tumorigenesis (Zhang *et al.* 2011). Overexpression of *FAF1* inhibits Wnt reporter activity by increasing the degradation of cytosolic β-catenin. The Wnt/β-catenin signalling system is one of the key pathways in the formation of cartilage and bone and their homeostasis (Kramer *et al.* 2010). The biological functions of *FAF1* may explain the association found here between a polymorphism in this gene and OC lesions in horses.

Although further validation is needed for the associations slightly under the significance threshold, these associations

Table 5 Significant (when F Reg > F Th) and suggestive associations between SNPs and different locations of OC lesions.

Locus	GenBank/dbSNP[1]	OC location	Mean	SD	F Th	Allele[2]	F reg	SE	P-value	Effect	Effect/SD
FAF1	rs68596812	Right MTP	0.347	0.108	8.657	G	9.119[3]	0.025	0.003	0.074	0.69
FCN3	g.8256C>T AAWR02028318	Right FP	0.309	0.053	8.020	T	7.104	0.012	0.009	0.031	0.59
COL1A2	rs69595660	Total	0.516	0.258	8.081	T	6.745	0.031	0.010	0.078	0.30

F Th, trait significant thresholds; F reg, F regression statistics; MTP, metatarsophalangeal; FP, femoropatellar; Total, sum of lesions in different locations per individual.

[1]GenBank accession numbers for *Equus caballus* sequences including the interrogated single nucleotide polymorphisms (SNPs) or dbSNP accession numbers.

[2]Allele positively correlated with the trait.

[3]Significant associations.

suggest the implication of the *FCN3* and *COL1A2* genes in the aetiology of OC in the Spanish Purebred horse breed. The *ficolin (collagen/fibrinogen domain containing) 3* gene (*FCN3*) has been previously associated with the presence of OC and OCD lesions in fetlock in Hanoverian Warmblood horses (Dierks *et al.* 2010). FCN3, also known as H-ficolin or Hakata antigen, is a complement-activating pattern recognition molecule, member of the ficolin/opsonin p35 family and present in serum (Michalski *et al.* 2015). FCN3 possesses an *N*-terminal cysteine-rich collagen-like domain that forms complexes with mannose-binding lectin-associated serine proteases and activates the lectin pathway (Matsushita *et al.* 2002). FCN3 seems to be an important component of innate immunity, showing a protective activity against bacterial pathogens (Michalski *et al.* 2015). Although the involvement of the immune response in OC pathogenesis remains unknown, it has been reported that the progression of OC lesions can lead to osteoarthritis (Scanzello *et al.* 2008; Wang *et al.* 2011), and different functional categories and canonical pathways related to immune responses have been recently implicated in the pathogenesis of OC (Rangkasenee *et al.* 2013c).

Finally, *COL1A2* is a component of type I collagen, which is found in most connective tissues, including cartilage, bone and tendon. The increase in the expression of type I collagen (*COL1A1*) in horses with clinic symptoms of OC has been attributed to a healing response (Semevolos *et al.* 2001). However, Mirams *et al.* (2009) found an increased expression of *COL1A1* in early lesions, suggesting that it may be a primary alteration that reflects an altered state on the differentiation of chondrocytes.

In conclusion, in this study, we identified three SNPs in the *FAF1*, *FCN3* and *COL1A2* genes as significantly or suggestively associated with OC in a sample of 144 Spanish Purebred horses. Given their biological functions, these loci seem suitable functional candidate genes for OC in this breed. All three polymorphisms were located in introns, and further resequencing of these genes will help to elucidate the causal mutation behind this association. Meanwhile, these SNPs could be used in a marker-assisted selection context to improve horse health, welfare and competitive lifespan. This study contributes to the ongoing efforts in identifying genes responsible for OC in horses.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional supporting information may be found online in the supporting information tab for this article:

Table S1 Forty-eight polymorphisms genotyped, multiplex and primer extension primers.