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Age-dependent expression of osteochondrosis-related genes in equine leukocytes

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ABSTRACT

Introduction: Osteochondrosis (OC) is a developmental disease in horses which has a significant impact on the horse's welfare and performance. The early disturbance in the process of endochondral ossification progresses to inflammatory and repair processes in older horses. Previously, differentially expressed genes in leukocytes of OC-affected horses have been identified. The aim of the present study is to detect age-related changes in these differentially expressed genes.

Materials and Methods: The expression of OC-related genes was analysed by real-time PCR and subsequent statistical analysis (ΔΔCT) in the leukocytes of 135 Belgian Warmblood horses divided into three different age groups: <12 months (n=47), 18–24 months (n=50) >30 months (n=38).

Results: Relative expression of genes of horses less than 12 months of age showed significant induction of the genes MGAT4A, PRKCG, MHCI, ApoB, ApoB3G, B4GALT6 and a significantly lower expression of the genes OAS3. Horses of 18–24 months of age, showed a significantly higher expression of the genes TBC1D9, MGAT4A, IFIH1, MHCIIa and MMP1. Horses of more than 30 months of age showed a significantly higher expression of the genes MGAT4A, HP, SECTM1 compared with their age-matched control groups.

Conclusions: The study demonstrates that OC-related genes are differentially expressed in horses of different ages compared with their age-matched controls. Some of the genes may be implicated in cell signalling and differentiation as well as carbohydrate and lipid metabolism and inflammation. However, the causal relationship between the differentially expressed genes and the development and progression of the OC lesions needs to be determined.

INTRODUCTION

Osteochondrosis (OC) is a developmental disease of growing horses. It is defined as a disturbance in the process of endochondral ossification (Rejnö and Strömberg 1978). The disturbance can lead to the formation of detached fragments (osteochondrosis dissecans (OCD)), fissures, or subchondral bone cysts at the epiphyseal growth cartilage (Rejnö and Strömberg 1978, Ytrehus and others 2007). OCD is a frequent cause of pain, lameness and reduced performance in young athletic horses (McIlwraith 1993, Verwilghen and others 2013).

The disease appears to be multifactorial in origin, including skeletal growth rates, nutrition, endocrinological factors, exercise, biomechanics and genetic effects (Jeffcott 1991). Several studies have demonstrated differentially expressed genes like, for example, Indian hedgehog (Ihh), transforming growth factor β 1 (TGF-β 1), Gli1 and insulin-like growth factor I (IGF-I) parathyroid hormone-related peptide (PTH-rP) in the cartilage of OC-affected horses (Semevolos and others 2001, 2002, 2005, Zabek and others 2002).

In a previous study, we analysed the transcript profile of leukocytes from horses affected with OC using a high throughput sequencing method (Serteyn and others 2010). Metabolic pathway analysis showed an obvious dysregulation of several signalling pathways related to cartilage formation and cartilage repair, such as Wnt, Indian hedgehog and TGF-β signalling pathways. Other genes that were differentially regulated may play a role in high-carbohydrate diet, abnormal insulin metabolism or inflammation (Serteyn and others 2010). Horses of this study had a mean age of 2.5 years and were relatively old regarding the origin of the disease. OC occurs early in the age of the horse (Lecocq and others 2008). The lesions undergo a variety of processes until they become visible as radiographic finding. Thereafter they may become modified by regenerative or degenerative processes (Jacket and others 2013). The global aim of our research is to find biomarkers of samples which can be easily obtained. The suitability of leukocytes for gene profiling is related to their active metabolism and accessibility by simple venipuncture (Liew and others

2006). The aim of the present study is to investigate the leukocyte gene expression of horses of different age groups with OC lesions.

MATERIALS AND METHODS

One hundred and thirty-five Belgian Warmblood horses were selected for this study, which followed a 'case control study' model. Selection was made on the base of the undoubtable results of the radiographies among the horses presented at the European Centre of the horse (Vielsalm, Belgium) by the breeders in the context of an orthopaedic diseases screening programme. OC-affected horses were classified into three different age groups: <12 months (AGE 1, n=35), 18–24 months (AGE 2, n=38), >30 months (AGE 3, n=26). Furthermore, 36 horses (AGE 1: n=12, AGE 2: n=12, AGE 3: n=12) without any evidence of OC lesions were selected and analysed as control groups.

All horses were sedated for the radiographic examination using detomidine (0.01 mg/kg intravenously) alone or combined with butorphanol (0.02 mg/kg intravenously). The following views were taken: lateromedial views of the four fetlocks, lateromedial and plantarolateral-dorsomedial oblique views of the hocks and a lateromedial view of the stifles. Horses were diagnosed on the basis of characteristic lesions of abnormal endochondral ossification located in the metacarpo and metarso phalangeal tibiotarsal, and femoro-patellar joints (Denoix and others 2013). Concerning the fetlock joint, fragmentation and irregularity observed on the dorsal aspect of the sagittal ridge and the condyles of the metacarpus or metatarsus were considered as OC lesions. Horses with dorsal, palmar or plantar fragmentations at the margin of the proximal phalanx were not included in this study. Concerning the hock, OC lesions were identified as fragments still in place (intermediate ridge) or surface irregularities of the trochlear ridge(s) or malleolus. Concerning the stifle joint, variably sized irregularity or flattening of the lateral trochlear ridge of the femur with sometimes partial calcification of the tissue within the defect were considered as OC lesions.

Horses with osteoarthritis and other radiological abnormalities, which were not clearly OC lesions, were excluded from this study. Blood was collected from each horse with PAXgene blood RNA tubes (BD Diagnostics, Le Pont de Claix, France) and frozen at −80°C for later analysis.

Total RNA was extracted from whole blood (2.5 ml) using the PAXgene blood RNA kit (Qiagen, Courtaboeuf, France). The integrity of total RNA extracted using the PAXgene method was checked by capillary electrophoresis analysis on Agilent BioAnalyser 2100 (Agilent, Palo Alto, California, USA). RNA quantity was measured using a spectrophotometer NanoDrop ND-1000 (Thermo Scientific, Les Ulis, France).

cDNA was synthesised using ~300 ng of total RNA and performed, using the VILO reverse transcription Kit, according to the manufacturer's protocol (Invitrogen, Cergy Pontoise, France).

Pre-amplification was performed with a 22 pooled (final 0.2× of each) SYBR Green assay. After pre-amplification PCR, the product was treated with 4 U of Exonuclease I (NEB) then diluted 1:5 with 1× TE Buffer and stored in −80°C until needed. Quantitative PCR was carried out using the 96.96 dynamic array (Fluidigm Corporation, California, USA) following the manufacturer's protocol. Specifically, a 5 µl sample mixture was prepared for each sample containing 1× TaqMan Gene Expression Master Mix, 1× GE Sample Loading Reagent (Fluidigm PN 85000746), 1× EvaGreen (Interchim) and each of diluted pre-amplified cDNA. 5 µl of Assay mix was prepared with 1× each of SYBR Green assay and 1× Assay Loading Reagent (Fluidigm PN 85000736). An IFC controller was used to prime the fluidics array (chip) with control line fluid and then with samples and assay mixes in the appropriate inlets. After loading, the chip was placed in the BioMark Instrument for a first step of thermal mixing at 50°C for two minutes, 70°C for 30 minutes and 25°C for 10 minutes, then UNG & HotStart step at 50°C for two minutes followed by 95°C for two minutes. PCR was performed by 35 cycles at 95°C for 15 seconds and 60°C for one minute. Finally, melting curve was performed by increasing temperature from 60 to 95°C. The data were analysed with Real-Time PCR Analysis Software in the BIOMARK instrument (Fluidigm Corporation, California, USA).

RNA level quantification was assessed by calculating $2^{-\Delta\Delta CT}$ (Schmittgen and Livak 2008).

In the previous study, we identified 2553 genes significantly upregulated or downregulated between the OC group and the control group (Serteyn and others 2010). For this study, we have chosen 34 genes following different criteria: a low P value (inferior to 0.1 per cent), fold induction superior to 5, quality of annotation (existence of a lot of predicted mRNA in the horse genome), and a mix of upregulated and downregulated genes membership of a known signalling pathway (Table 1). Four control genes: WARS (tryptophanyl-tRNA synthetase), RIGE (retinoic acid induced gene E protein), B2M (β-2 microglobulin) and TUBB2C (tubulin, β 2C), referred to as housekeeping genes, were used to normalise mRNA levels between different samples. These four control genes were defined in a previous transcriptomic study (Serteyn and others 2010).

Statistical analysis was performed according to Yuan and others (2008) using the data of control groups as references values (ΔΔCT) respectively for each AGE. Then, the significance analysis of microarrays (SAM) method was used to compare the levels of gene expression as a marker of OC, taking into account the age effect between control samples and study samples. We used the corrected P value based on the false discovery rate (FDR) method (Tusher and others 2001). The results are expressed for each gene as a ratio between the expression levels in the OC-affected group versus the

TABLE 1: List of the 33 genes selected on several criteria from Serteyn *et al.* (2010): a low P value (inferior to 0.1 per cent), fold induction superior to 5, quality of annotation, a mix of upregulated and downregulated genes membership of a known signalling pathway

Gene symbol	Name	Transcript ID (Ensembl or Genbank)
ADAMTLS4	ADAMTS-like protein 4 precursor	ENSECAT00000020422
ApoB	Apolipoprotein B	ENST00000233242
ApoB3G	similar to ApoB mRNA editing enzyme catalytic polypeptide-like 3G	XM_001916520
B4GALT6	β-1,4-galactosyltransferase 6	ENSECAT00000018745
BMP5	Bone morphogenetic protein 5	ENSECAG00000007138
CLK1	Dual specificity protein kinase	ENSECAT00000016444
CRKL	Crk-like protein	ENSECAT00000018670
CSNK1E	Casein kinase I isoform epsilon	ENSECAT00000023450
CtBP1	C-terminal-binding protein 1	ENSECAT00000016987
CUL5	Cullin-5 (vasopressin-activated calcium-mobilising receptor)	ENSECAT00000026414
DVL1	Segment polarity protein dishevelled homologue DVL-1	ENST00000378891
DVL3	Segment polarity protein dishevelled homologue DVL-3	ENSECAT00000009739
FOXL1	Forkhead box L1	ENST00000320241
FOXO1	Forkhead box O1	ENST00000379561
FZD1	Frizzled family receptor 1	ENSECAG00000001326
GRB2	Growth factor receptor-bound protein 2	ENSECAG00000006524
GSK3B	Glycogen synthase kinase 3 β	ENSECAT00000026913
Hp	Haptoglobin	XM_001497810
IFIH1	Interferon induced with helicase C domain	ENST00000263642
IKBKB	Inhibitor of nuclear factor κ-B kinase subunit β	ENSECAT00000005219
ISG15	Interferon-induced protein precursor	ENSECAT00000001183
MGAT4A	Mannosyl glycoproteinacetylglucosaminyltransferase	ENSECAG00000015468
MHCI	MHC class I heavy chain	ENSECAT00000021999
MHCIIa	Similar to MHC class II antigen DQ α chain	ENSECAT00000022398
MMP1	Interstitial collagenase precursor (Matrix metalloproteinase-1)	ENSECAT00000025715
OAS3	2′-5′-oligoadenylate synthetase 3	ENST00000549918
PPP1CB	Protein phosphatase 1, catalytic subunit, β isoform	ENSECAG00000009359
PPR2A	Protein phosphatase 2, regulatory subunit A, α isoform	ENSECAT00000019754
PRKCG	Protein kinase C, γ	ENSECAT00000020980
PYGL	Phosphorylase, glycogen, liver	ENSECAT00000013037
RUSC2	Iporin (interacting protein of Rab1) (RUN and SH3 domain-containing protein 2)	ENSECAT00000026463
SECTM1	Secreted and transmembrane protein 1 precursor	ENSECAT00000006039
SMAD5	SMAD family member 5	ENSECAG00000000815
TBC1D9	TBC1 domain family member 9B	ENSECAT00000018936
WASH1	WAS protein family homologue 1	XM_001493185.2

healthy horses. Our statistical criterion for the SAM analysis was the following: only the genes with a level of expression strictly below 0.7 or superior to 1.5 and satisfying at least a FDR strictly lower than 5 per cent were considered as possible biomarkers.

RESULTS

The results of the SAM analysis were reported in Table 2 for each age group. We found several biomarkers satisfying our biomarker selection criterion.

Relative expression of genes of horses less than 12 months of age showed significant induction of the genes MGAT4A (mannosyl-glycol-protein acetylglucosaminyltransferase), PRKCG (protein kinase C, gamma), MHCI (MHC class I, α chain), ApoB (apolipoprotein B), ApoB3G (similar to apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G), B4GALT6 (β-1,4-galactosyltransferase 6), the three latter genes were above the golden threshold of two-fold change. Further, there was a significantly lower expression of the gene OAS3 (2′5′-oligoadenylate synthetase 3) with a two-fold change.

Horses of 18–24 months of age, showed a significantly higher expression of the genes TBC1D9 (TBC1 domain family, member 9 (with GRAM domain)), MGAT4A, IFIH1 (interferon-induced helicase C domain-containing protein 1), MHCIIa (similar to MHC class I, α chain) and MMP1 (interstitial collagenase precursor), withIFIH1 and MMP1 above the two-fold change for upregulated genes.

Horses of more than 30 months of age showed a significantly higher expression of the genes MGAT4A, HP (haptoglobin), SECTM1 (secreted and transmembrane protein 1 precursor (protein K12)), MHCIIa, PYGL (phosphorylase, glycogen, liver), CUL5 (cullin-5,

TABLE 2: SAM-plot results with the overexpressed an underexpressed genes

	<12 months		18–24 months		>30 months	
	Ratio	FDR	Ratio	FDR	Ratio	FDR
MGAT4A	**1.808**	0.0	**1.508**	0.0	**1.76**	3.2
PRKCG	**1.83**	0.0				
MHCI	**1.592**	0.0				
ApoB	**2.965**	0.0				
ApoB3G	**4.768**	0.0				
B4GALT6	**2.185**	0.0				
OAS3	*0.428*	*0.0*			*0.648*	*0.0*
TBC1D9			**1.89**	0.0		
IFIH1			**2.35**	0.0		
MHCIIa			**1.532**	0.0	**1.607**	3.2
MMP1			**3.225**	0.0	**3.863**	3.2
HP					**3.072**	3.2
SECTM1					**3.301**	0.0
PYGL					**1.729**	3.2
CUL5					**1.812**	3.2
GSK3B					**1.519**	3.2
WASH1					*0.573*	*0.0*

The Ratio (<0.7 and >1.5) and the FDR (<5 per cent) are reported with the overexpressed (bold) and underexpressed (italic) genes. The FDR is a corrected p value (Tusher *et al.* 2001)
SAM, significance analysis of microarrays

vasopressin-activated calcium mobilising receptor), GSK3B (glycogen synthase kinase 3 β) and MMP1. Again we found MMP1 above the golden two-fold change threshold. Two other genes are worth mentioning: SECTM1 and HP. For the downregulated genes a significant lower expression of OAS3 and WASH1 (WAS protein family homologue 1) were found by the SAM analysis.

Based on our very low FDR rates we computed Fig 1 which combines in a Venn diagram the common markers between the groups. We found several common markers such as MGAT4A which is upregulated at all ages and could be seen as our 'supermarker', OAS3 as a common disease marker for groups of horses less than 12 months of age and horses of more than 30 months of age, and two common markers MHCIIa and MMP1 for horses of 18–24 months of age and horses of more than 30 months of age.

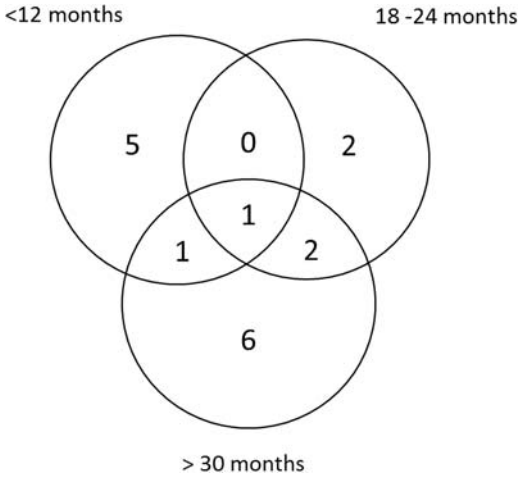

FIG 1: Venn diagram showing the number of common markers between the three age groups

DISCUSSION

We previously demonstrated that leukocytes of affected horses showed differential expression of genes related to several metabolic pathways, for example, Wnt signalling, insulin signalling, TGF-β signalling and Ihh signalling (Serteyn and others 2010). Mature leukocytes have been shown to express a variety of mRNAs and they are able to respond to external stimuli by rapid and complex changes in gene expression (Bertrand and others 2004). Likewise, Liew and others (2006) considered peripheral blood leukocytes as a surrogate tissue to substitute for traditional tissue specimens that are not easily accessible. The suitability of leukocytes for gene profiling is related to their active metabolism and accessibility by simple venipuncture, which may allow a repeated time-series analysis of changes in gene expression in response to changing environmental or disease factors (Liew and others 2006). In horses, Kamm and others (2013) used a similar method to study the transcript profile of leukocytes from horses affected by osteoarthritis.

Histology studies (Laverty and Girard 2013) and cartilage-derived transcriptome studies have demonstrated timely changes in the initiation and the progression of the cartilage lesions. The initial step in the pathogenesis is one or several of the following events: formation of a fragile cartilage, failure of chondrocyte differentiation, subchondral bone necrosis, and failure of blood supply to the growth cartilage (Ytrehus and others 2007, Olstad and others 2013). In the more chronic stages of the disease, secondary degenerative and regenerative processes overshadow the initial events (Bertone and others 2005). The timely fashion of the events is also reflected in the transcriptome changes of leukocytes of OC-affected horses compared with age-matched OC-free controls. However, the transcript level of the MGAT4A gene remains significantly higher in the OC-affected horses of all ages. This gene is implicated in the intracellular transport of glucose via the membrane localisation of GLUT2 (Johswich and others 2014). The MGAT4A transcript level is significantly higher in white blood cells of peripheral blood of human patients affected by type 2 diabetes (T2D) (Lopez-Orduña and others 2007). They suggest that high levels of glucose and triglycerides could induce the high MGAT4A transcript levels, especially in subjects with a particular T2D genetic background. A similar explanation could be proposed for the OC-affected foals. In an animal model, transient hyperglycaemia causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. This mechanism implicates a mitochondrial pathway with a high superoxide anion production

(El-Osta and others 2008). Recently, abnormal mitochondria and endoplasmic reticulum were observed in the deep zone of OC cartilage (Desjardin and others 2014). The mitochondrial dysfunction could be partially explained by the altered Wnt signalling pathway observed in the OC-affected horses because this pathway is known as a key regulator of mitochondrial function (Serteyn and others 2010, Yoon and others 2010).

Other genes identified as possible biomarkers in this study are related to the carbohydrate and lipid metabolism (APOB, TBC1D9, B4GALT6, PYGL, GSK3) or gene expression, cell growth, apoptosis and differentiation (APOB3G, PRKCG, CUL5, SECTM1, WASH1) or related to inflammatory and immune mechanisms (OAS3, MMP1, HP, IF1H1, MHCI, MHCIIa).

Their role in the pathogenesis of equine OC remains to be determined, but a high-energy diet is known to induce OC lesions in foals (Savage and others 1993, Mirams and others 2009). These observations were illustrated recently by Vander Heyden and others (2013). It appears that mares fed with concentrates during gestation are more likely to produce foals that are subsequently affected by OC compared with other mares.

Even though the real-time PCR analysis of the 135 horses is conclusive, the potential markers should be subjected to individual analysis in order to validate their clinical potential. Limitations of this study include the fact that neither the affected joint nor other lesions of endochondral ossification such as subchondral cysts have been considered.

Even if the causal relationship between the differentially expressed genes and the development and progression of the OC lesions needs to be determined, this study clearly demonstrates that OC-affected horses show particular changes in their leukocyte transcriptome and that these modifications change with age.

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