Biological processes related to ribosome and mitochondrial functions might be involved in the osteochondrosis latens manifestation in gilts

A.M.G. Ibelli[1], J.O. Peixoto[1], I.R. Savoldi[2], K.B. Carmo[2], N. Morés[1], M A.Z. Morés[1], M.E. Cantão[1], L. L. Coutinho[3]& M. C. Ledur[1]

[1]Embrapa Suínos e Aves, caixa postal 321, 89715-899, Concórdia, Santa Catarina, Brazil, adriana.ibelli@embrapa.br (Corresponding Author)
[2]Universidade do Contestado, Rua Victor Sopelsa, 3000, 89700-970, Concórdia, SC, Brazil
[3]Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Av. Pádua Dias, 11, 13418-900, Piracicaba, São Paulo, Brazil

Summary

The articular osteochondrosis (OC) is a disturbance of endochondral ossification occurring in humans and livestock animals. In pigs, OC is one of the major causes of leg weakness, leading to economic losses and reducing the animal welfare. The aim of this study was to evaluate the differential gene expression related to OC *latens* in femoral articular cartilage in swine. Thus, samples of MS115 5-months-old gilts, normal (control group) and affected with OC *latens* (affected group), were collected and submitted to histopathological and gene expression analysis. A total of 1,734 genes was differentially expressed (DE) between the two groups. Several biological processes (BP) were enriched including those already known as involved with OC, such as hypoxia, cartilage and bone development. Furthermore, new BP related to initiation of transcription and ribosome biogenesis were associated to this condition. The disruption of mitochondria and ribosome functions may contribute to the development of cartilage and bone disorders, including osteochondrosis and osteoarthritis. The activation of transcription factors (TFs), required to the cartilage and chondrocyte maturation since embrionary life, might also affect the expression of many extracellular matrix genes related to cartilage. The downregulation of TFs *ATOH8* and *SOX5* in the affected group would delay the chondroblast differentiation, hindering the cartilage formation and endochondral ossification. Besides the DE genes, three genes (*DAG1, SC16A* and *SPAG9*) had different isoforms expressed between the normal and affected groups. In this study, new genes and biological processes were described to be possibly involved in the manifestation of OC *latens* in gilts.

Keywords: gene expression, lameness, leg weakness, locomotor problems, RNA-Seq, swine

Introduction

The articular osteochondrosis (OC) is a disturbance of endochondral ossification occurring in humans and livestock animals (Etterlin *et al.*, 2017). In pigs, this disorder is one of the major causes of leg weakness and lameness, affecting up to 80% of the animals, which lead to economic losses and reduced animal welfare (Etterlin *et al.*, 2015). This condition is characterized by ischemic necrosis and chondronecrosis of epiphyseal growth cartilage (Ytrehus *et al.* 2007, Finnoy *et al.*, 2017) and, although it is commonly observed in swine, its etiology remains unclear. It has been reported that the OC lesions appear after birth, but it can have a period of regression, being stabilized after the fifth month of age (Serteyn *et al.*,

2014). Some findings suggest a genetic component involved with its development (Finnoy *et al.*, 2017), with heritability estimates ranging from 0.29 to 0.49 (Aasmundstad *et al.*, 2013, Aasmundstad *et al.*, 2015). Also, QTLs and candidate genes have already been identified for OC in pigs (Rangkasenee *et al.*, 2013). During the maturation, the articular cartilage suffers naturally transcriptional changes related to inflammatory responses to injury and vascularization, which can affect the cartilage ability to regenerate (Abapala & Kim, 2017), leading to the development of cartilage disorders. Therefore, the differential gene expression based on RNA-Seq data was evaluated between normal MS115 gilts and those affected with OC *latens* to identify genes possibly involved in this disorder.

Material and Methods

Animals and sample collection

For this study, 15 lameness and 15 normal MS115 gilts (5 months of age) from the Embrapa Swine and Poultry National Research Center, Concórdia, SC, Brazil, were used. The gilts were raised in a standardizing system for swine with the management conditions following the recommendations for this synthetic male line and were supplied with water and feed *ad libitum*. Samples of the distal femoral articular cartilage (AC) were collected in a slaughterhouse for histological and gene expression analysis. The sample collection was according to the ethical guidelines of the Embrapa Swine and Poultry Ethics Committee on Animal Utilization, under the protocol number 011/2015.

Histopathological analysis

The histopathological analysis was performed to identify normal and osteochondrosis affected gilts. For this, axial sections of femur condyles were fixed in 10% buffered formalin and decalcified with formic acid-sodium citrate solution. Then, the histological sections were routinely processed for histopathology for observation of subchondral bone, stained with hematoxylin and eosin and visualized in light microscopy.

RNA extraction, library preparation and sequencing

For the gene expression analysis, 6 animals, 3 with OC *latens* (affected group) and 3 normal (control group) were selected based on histological classification. Approximately 100 mg of frozen femoral AC tissue had the total RNA extracted using Trizol© (Life Technologies), according to manufacturer´s protocol, followed by a RNA cleanup using the RNeasy mini kit (Qiagen). The RNA concentration was measured using Biodrop spectrophometer (Biodrop) and its integrity with Bioanalyzer 2100 equipment (Agilent). The libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina), with 2 μg of total RNA following the manufacturer´s protocol. The sequencing was performed in Illumina HiSeq 2500 sequencer (lllumina, USA) using 2x100bp paired-end reads at the Functional Genomics Center, ESALQ-USP, Piracicaba, São Paulo, Brazil.

Data analysis and functional annotation

The data quality control (QC) was performed in the SeqyClean software (https://github.com/ibest/seqyclean). Sequenced reads of each sample were mapped against the swine reference genome (Sscrofa 11.1, Ensembl release 90) using the STAR software

(Dobin *et al.*, 2013). Reads counting were performed in the HTseq-count script (Anders *et al.*, 2015). The differential expressed (DE) genes were obtained using the edgeR package (Robinson *et al.*, 2010) and genes were considered DE when false discovery rate (FDR) was ≤0.05. Negative and positive fold-changes indicate, respectively, down and upregulation of genes in the OC affected group compared to the control group. Furthermore, isoform analysis was carried out using the RSEM software (Li and Dewey, 2011). The FASTQ files were deposited in the SRA database (Bioproject # PRJNA349171). The functional annotation of the DE genes was performed using BioMart (https://www.ensembl.org/biomart/) and DAVID 6.8 database (https://david.ncifcrf.gov/) to obtain the gene ontology (GO) terms. The Revigo online tool (http://revigo.irb.hr/) was used to reduce the redundant GO terms. A gene interaction analysis was carried out with the STRING database (https://string-db.org/).

Results and discussion

The femur histopathological analysis from 30 collected gilts showed that 17 presented OC *latens* histological lesions, 6 were normal and 7 presented an inconclusive diagnostic. These data highlight the complexity of the osteochondrosis clinical diagnosis, as reported in the literature (Ytrehus *et al.*, 2007). The main histological alterations observed in the distal femur were islands of non-mineralized cartilage in the primary spongiosa, persistent non-mineralized cartilage in the primary spongiosa with areas of necrosis and mild synovial proliferation.

Regarding the transcriptome analysis, about 24 million paired-end reads were kept after the QC, being 97% uniquely mapped across the swine reference genome. Out of those, 83.11% were mapped in genes. A total of 11,953 genes was expressed in the swine femoral articular cartilage, being 1,734 DE between the affected and control group. According to the functional annotation, 1,145 genes were present in the DAVID 6.8 database, comprising 557 biological processes (BP) based on GO analysis, such as those related to skeletal system, hypoxia, cartilage and bone development, which have already been described as related to osteochondrosis (Etterlin *et al.*, 2017). Also, several BP enriched in our study have not been previously associated with OC *latens* in pigs, such as regulation of ribosomal maturation and translation, macroautophagy and TOR signaling. These processes grouped genes with basal functions in the cell, such as the initiation of transcription and ribosome biogenesis (Nakhoul, *et al.*, 2014). About 20 mitochondrial ribosomal protein genes (i.e. *MRPL, MRPS*) and 45 ribosomal protein (i.e. *RPL, RPS, RR*) genes were downregulated in the affected group, and this pattern might be involved with the disruption of mitochondria and ribosome functions. It has been suggested that those mechanisms facilitate cartilage and bone disorders, including osteochondrosis in horses and osteoarthritis in humans, through disrupting chondrocyte responses to mechanical stimuli and increasing the reactive oxygen species (ROS) (Desjardin *et al.*, 2014, Trainor and Merril, 2014, Kapitanov *et al.*, 2017). Chondrocyte differentiation (*SCRG1*) and ROS (*SOD3*) related genes were also DE between groups, reinforcing the importance of the BP discovered in this study. In addition, the activation of transcription factors (TFs), required to the cartilage and chondrocyte maturation since embrionary life, might affect the expression of many extracellular matrix genes related to cartilage differentiation. The TFs downregulation, as observed here for *ATOH8* and *SOX5* genes, would delay the chondroblast differentiation, hindering the cartilage growth plate formation and endochondral ossification (Smits *et al.*, 2001). The set of candidate genes discussed may be directly involved in the observed histological lesions due to their basal functions. Besides the DE genes, 3 genes had different isoforms expressed between the studied groups: *SPAG9*

(sperm associated antigen 9), *DAG1* (Alpha-dystroglycan) and *SC16A* (SEC16 endoplasmic reticulum export factor), presenting functions in angiogenesis, connecting the extracellular matrix to the cytoskeleton and in protein transport from the endoplasmic reticulum, respectively. The presence of differentially expressed isoforms between the normal and affected groups of animals suggests that alternative splicing might be involved in the OC *latens* development.

Conclusions

The differential expression analysis of distal femoral articular cartilage from normal and OC *latens* affected gilts revealed new genes and biological processes related to the occurrence of OC *latens.* The disruption of mitochondria, TFs and ribosome related genes might be involved with predisposition to the development of OC *latens* in gilts.

Acknowledgements

This study was supported by the CNPq project # 480575/2013-4. LL Coutinho is a CNPq fellow. I.R. Savoldi and K.B. Carmo received a PIBIC/CNPq scholarship.

List of References

Aasmundstad, T., I. Andersen-Ranberg, Ø. Nordbø, T. Meuwissen, O. Vangen & E. Grindflek, 2015. The effect of including genomic relationships in the estimation of genetic parameters of functional traits in pigs.Anim. Breed. Genet.132:386–39.

Aasmundstad, T., J. Kongsro, M. Wetten, N.I. Dolvik & O. Vangen, 2013. Osteochondrosis in pigs diagnosed with computed tomography: heritabilities and genetic corre- lations to weight gain in specific age intervals. Animal. 7:1576-82.

Adapala, N.S. & H.K. Kim, 2017. A genome-wide transcriptomic analysis of articular cartilage during normal maturation in pigs. Gene. 627:508-518.

Anders, S. & W. Huber, 2010. Differential expression analysis for sequence count data. Genome Biology, 11:R106–R106.

Desjardin, C., S. Chat, M. Gilles, R. Legendre, J. Riviere, X. Mata, T. Balliau, D. Esquerré, E.P. Cribiu, J.M. Betch & L. Schibler, 2014. Involvement of mitochondrial dysfunction and ER-stress in the physiopathology of equine osteochondritis dissecans (OCD). Exp Mol Pathol. 96(3):328-38.

Dobin, A., C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson & T. R. Gingeras, 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics, 29:15–21.

Etterlin, P.E., D.A. Morrison, J. Österberg, B. Ytrehus, E. Heldmer & S. Ekman, 2015. Osteochondrosis, but not lameness, is more frequent among free-range pigs than confined herd-mates. Acta Vet Scand. 2015:57:63.

Etterlin, P.E., S. Ekman, R. Strand, K. Olstad & C.J. Ley, 2017. Osteochondrosis, Synovial Fossae, and Articular Indentations in the Talus and Distal Tibia of Growing Domestic Pigs and Wild Boars. Vet Pathol. 54(3):445-456.

Finnøy, A., K. Olstad & M.B. Lilledahl, 2017. Non-linear optical microscopy of cartilage canals in the distal femur of young pigs may reveal the cause of articular osteochondrosis. BMC Vet Res. 13(1):270.

Kapitanov, G.I., B.P. Ayati & J.A. 2017. Modeling the effect of blunt impact on mitochondrial function in cartilage: implications for development of osteoarthritis. PeerJ.

5:e3468.
Li, B. & N. Dewey, 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics, 12:323.
Nakhoul, H., J. Ke, S. Zhou, W. Liao, S.X. Zeng & H. Lu, 2014. Ribosomopathies: Mechanisms of Disease. Clin Med Insights Blood Disord. 2014. 7:7-16.
Rangkasenee, N., E. Murani, K. Schellander, M.U. Cinar, S. Ponsuksili & K. Wimmers, 2013. Gene expression profiling of articular cartilage reveals functional pathways and networks of candidate genes for osteochondrosis in pigs. Physiological Genomics. 45:856-865.
Robinson, M.D., D.J McCarthy & G.K. Smyth, 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26: 139-140.
Serteyn, D., D. Piquemal, L. Mendoza & F. Noguier, 2014. Osteochondrosis-Related Gene Expression in Equine Leukocytes Differs among Affected Joints in Foals. J Mol Biomark Diagn 5:189.
Smits, P., P. Li, J. Mandel, Z. Zhang , J.M. Deng, R.R. Behringer, B. de Crombrugghe & V. Lefebvre, 2001. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Dev Cell. 1(2):277-90.
Trainor, P.A. & A.E. Merrill, 2014. Ribosome Biogenesis in Skeletal Development and the Pathogenesis of Skeletal Disorders. Biochim Biophys Acta. 42(6):769-78.
Ytrehus, B., C.S. Carlson & S. Ekman, 2007. Etiology and pathogenesis of osteochondrosis. Vet Pathol. 44:429–448.