Expressão gênica diferencial envolvida com Condronecrose bacteriana com osteomielite em frangos de corte com 35 dias de idade

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Resumo: A incidência de problemas ósseos é considerada uma das principais preocupações para a indústria avícola devido a perdas econômicas significativas e ao impacto negativo no bem-estar. As vias metabólicas e os genes envolvidos nas patologias ósseas permanecem desconhecidos. A condronecrose bacteriana com osteomielite (CBO) é uma das principais doenças ligadas a problemas locomotores em frangos. Na tentativa de esclarecer os mecanismos genéticos envolvidos na manifestação da CBO, objetivou-se identificar os genes diferencialmente expressos no fêmur de frangos de corte normais e afetados por esta desordem, por meio da tecnologia de RNA-Seq. Neste estudo foram utilizados frangos de corte comerciais machos aos 35 dias de idade, sendo 4 normais e 4 com CBO inicial. O sequenciamento das bibliotecas foi realizado na plataforma *Illumina*. Os genes diferencialmente expressos foram selecionados utilizando-se o pacote EdgeR, com base no *False Discovery Rate* (FDR≤ 0,05) e log de *fold-change* ≥ 1,0. Um total de 11.500 genes se apresentaram expressos nesse tecido ósseo, dos quais 153 foram diferencialmente expressos entre frangos normais e afetados. Após a análise de ontologia gênica (GO), alguns genes candidatos foram prospectados. O conhecimento dos genes que controlam esse distúrbio pode apoiar estratégias de melhoramento para a produção de frangos de corte comerciais resilientes para CBO, com o objetivo de se reduzir as perdas ocasionadas por problemas locomotores na indústria avícola.

Palavras–chave: CBO, genes diferencialmente expressos, GO, RNA-Seq

Gene expression related to the Bacterial Chondronecrosis with Osteomyelitis in 35 day old Broilers

Abstract: Bone problems are considered one of the main concerns to the poultry industry due to the significant economic losses and the negative impact on welfare. The genetic pathways and genes involved in bone pathologies remains unclear. The Bacterial Chondronecrosis with Osteomyelitis (BCO) is one of the most important leg disorders in commercial broilers. In order to clarify the genetic mechanisms involved in BCO, the aim of this study was to identify differentially expressed genes in femur of normal and affected broilers with this disorder, using RNA-Seq technology. A total of 8 commercial male broilers with 35 days of age were used. Femoral samples were collected from 4 healthy animals and from 4 broilers with initial BCO. mRNA sequencing was performed using Illumina technology. Differentially expressed genes (DEG) were obtained using the edgeR package based on the False Discovery Rate (FDR≤0.05) and the log fold-change ≥ 1.0. A total of 11,500 genes were found to be expressed in the femur tissue. Of those, 153 DEG were identified between normal and injured broilers. After Gene Ontology (GO) analyses, some candidate genes were prospected. The knowledge of the genes that control this disorder could support breeding strategies for production of commercial broilers resilient to BCO to reduce losses caused by leg problems in the poultry industry.

Introduction

The poultry production system has focused in the intense selection for heavier and faster growing broilers (Havenstein et al., 2003). However, at the same time, the incidence of bone problems has increased and being considered one of the main concerns to the poultry industry due to the significant economic losses and the negative impact on welfare (Oviedo-Rondón, 2007). Some efforts to select animals against leg abnormalities have been done, but with insufficient progress. The genetic pathways and genes involved in bone pathologies remain unclear. Bacterial Chondronecrosis with Osteomyelitis (BCO) is one of the most important leg disorders in commercial broilers (McNamee, et al. 2000). This pathology is also known as Femoral Head Necrosis (FHN) and its etiology and genetic mechanisms involved are not completely understood. Attempting to clarify the pathways involved in

BCO, the aim of this study was to identify differentially expressed genes at the femur growth plates of normal and affected broilers at 35 days of age.

Material e Methods

Experimental animals and sampling: Eight commercial male broilers with 35 days of age, raised at the same flock from a commercial facility, were used in this study. Body condition score was evaluated in all the animals prior to the necropsy. This study was performed with the approval of the Embrapa Swine and Poultry Ethical Committee of Animal Use (CEUA) under protocol number 012/2012. The femur growth plates were classified according to the presence or absence of different levels of BCO, according to visual observation of compatible necrosis lesions. Femoral samples were collected from 4 healthy (non-affected group) and 4 affected broilers with initial BCO (affected group). After collection, samples were stored in liquid nitrogen and transferred to the -80ºC freezer until the RNA extraction.

RNA extraction: Total RNA was extracted from 100mg from femoral head tissue using Trizol Reagent® (Life Technologies), following the manufacturer's instructions. Total RNA was quantified in Nanodrop spectrophotometer and integrity was confirmed in a BioAnalyzer Agilent 2100. All samples presented RIN (RNA integrity number) values above 8.

mRNA Sequencing and transcriptome analyses: Barcoded Illumina sequencing libraries were obtained using the TruSeq™ RNA Sample Prep Kit v2. Sequencing was performed on an Illumina HiSeq2000 equipment (Illumina, Inc.; San Diego CA, EUA) following the 2x100bp paired-end protocol. Raw sequence reads were first preprocessed and mapped to the reference chicken genome using the BWA-MEM software (Li and Durbin, 2009). Counts of fragments per kilobase of gene per million were obtained using the HTseq software (Anders and Huber, 2010) and differentially expressed genes (DEG) identified using the edgeR package (empirical digital gene expression analyses using R, ROBINSON et al., 2010). The DEGs were selected based on the level of False Discovery Rate (FDR≤0.05). In addition, a log fold-change (LFC) ≥1.0 was used as a biological criterion. For an overview of the DEG functions, a functional classification based on gene ontology (GO) terms, from GO slim, was performed using PANTHER Overrepresentation Test version 9.0 (Mi et al., 2013).

Results and Discussion

High-throughput RNA sequencing was used to generate a whole characterization of the femur head transcriptome. Approximately 15 million reads/sample were generated with 2x100 bp paired-end reads using Illumina HiSeq 2000 technology. After data cleaning, 97.5% of the reads were mapped to the chicken genome reference (Galgal4) comprising 17,108 genes (Ensembl release 75). A total of 11,500 genes were expressed in this femur tissue. Out of those, 153 were DEG between normal and injured broilers using the criteria FDR ≤ 0.05 and LFC ≥ 1.0 (Figure 1).
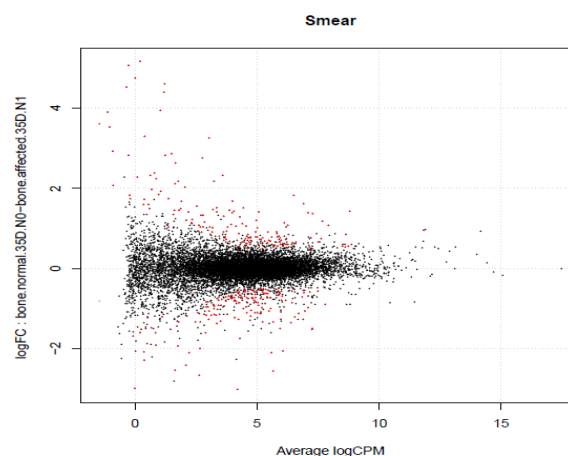

Figure 1. EdgeR smear plot showing differentially expressed genes (red).

PANTHER Overrepresentation Test identified 9 biological processes significantly (P<0.05) overrepresented in the DEG list: complement activation, cell adhesion, biological adhesion, mesoderm development, response to stimulus, system development, multicellular organismal process, single-multicellular organismal process and developmental process. One of the genes involved in those biological processes include *HSPB1*, a member from vascular endothelial growth factor (VEGF) signaling pathway, responsible to increase blood vessels invasion in the growth plate and cartilage.

The suppression/downregulation of VEGF factors have been directly associated with femoral head necrosis in many species (Varoga et al. 2009; Packialakshmi, 2015) since they act in antiapoptotic mechanisms on vessels. Lower VEGF expression decreases the blood vessels remodeling and angiogenesis in the femur, affecting bone formation, as these two pathways are intrinsically coupled (Blumer; Longato; Fritsch, 2008). It is important to highlight that the *HSPB1* gene, differentially expressed in this study, is included in the VEGF and angiogenesis pathways. Besides that, the lack of vascularization can modify the synovial fluid glucose and lactate, inducing an anaerobic metabolism (Huffman et al., 2006). This environment condition might facilitate the bacteria growth, which could lead to the incidence of BCO.

Other pathway involved in BCO found in our study was the inflammation mediated by chemokine and cytokine signaling pathway, being represented by *COL14A1* and *CCLI8* genes. These genes play an important role in collagen and osteogenesis and immunological processes, respectively (www.genecards.org/). However, no information is available on the correlation of these specific genes and the BCO condition. Moreover, the balance of many collagen genes is important to bone resorption and cartilage formation (Sitara; Aliprantis, 2010). The chemokines, such as *CCL18*, have many functions related to leukocyte activation during inflammatory responses. Recent studies have shown that the *CCL18* upregulation on articular cartilage and synovial tissue is involved with osteoarthritis (Zhou, Chen, Yang, 2015). Furthermore, this gene is induced by staphylococcal enterotoxins (www.genecards.org), one of the main bacteria associated with this disease (Jiang et al., 2015).

Conclusions

Genes with potential roles in the necrosis process related to BCO were identified. However, it is necessary further functional analysis on these genes to elucidate their processes. The knowledge of the genes that control BCO might support breeding strategies for production of commercial broilers resilient to BCO to reduce the negative impacts of this disorder on poultry production and welfare.

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