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VARIABILITY OF SELECTED GENES IN RELATION TO THE PARAMETERS OF BONES IN LAYING HENS: A PILOT STUDY

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ABSTRACT

The aim of the present study was to screen potential key genes associated with osteoporosis in laying hens. We performed association analysis of single nucleotide polymorphisms of selected genes encoding non-collagenous proteins (*IBSP, SPP1, FN1, MEPE, DCN, THBS2)* with parameters of bones in a group of ISA Brown laying hens. The surveyed parameters were bone breaking strength, length, width and bone mass. In this pilot study, nineteen samples for genes encoding non-collagenous proteins were tested. The polymorphisms were detected using PCR method and sequencing. Seven polymorphisms have been discovered in genes encoding non-collagenous proteins in the genes *FN1* (c.7413A>G, c.7440+57G>A, c.7440+105G>A, c.7441-71T>C), *DCN* (c.158C>T) and *SPP1* (c.362+117G>C, c.363-88G>C), of which two of these polymorphisms were synonymous and five were in the intron. Despite the position of the found polymorphisms, no association with the mechanical parameters of the bones were found.

Keywords: osteoporosis, laying hens, polymorphism, bone, non-collagenous proteins

INTRODUCTION

The balance between processes in which osteoclasts continuously break down old bone and osteoblasts form new bone plays an important role in maintaining bone mass and skeletal strength (**Costantini and Mäkitie, 2016**). The characteristic microarchitectural deterioration that threatens bone strength and is accompanied by increased fracture risk is typical of an imbalance in the regulation of bone remodeling (**Yang *et al.*, 2013**). This is the key pathophysiological mechanism of osteoporosis (**Li *et al.*, 2016**). Osteoporosis has been described histologically as a decrease in the volume of structural cancellous and cortical bone within the skeleton and being defined as local or systemic deficiency in the quantity of fully mineralized structural bone (**Cransberg *et al.*, 2001**). It is a complex disorder that is affected by number of factors including nutrition, sex, age, exercising, genetics and disease (**Guo *et al.*, 2017; Rocha-Braz and Ferraz-de-Souza, 2016**).

Skeletal problems in hens caused by osteoporosis affect both welfare and economic aspects of production. Important role in the development of the skeletal system plays genetic composition, so an alternative to reducing this problem could be the research of polymorphisms for marker-assisted selection (MAS) (**Fornari *et al.*, 2012**). Non-collagenous proteins (NCPs) are integral components of bone extracellular matrix (ECM) and exhibit multifunctional roles. These roles are critical for the bone resistance to fracture and bone quality. It has also been shown that by regulating the activity of osteoblast and osteoclast they affect bone modeling and subsequently alter their bone mass geometry. These proteins also affect mineralization of bone matrix, a key determinant of matrix quality and bone mechanical properties. NCPs impact hydroxyapatite crystallinity, formation of collagen fibrils and coordinate cell-matrix interactions. Therefore, by the effect of NCP on mineralization, they can affect the properties of bone material, such as hardness (**Bailey *et al.*, 2017; Morgan *et al.*, 2015; Nikel *et al.*, 2013**).

Osteopontin (SPP1), integrin-binding sialoprotein (IBSP) and extracellular phosphoglycoprotein matrix (MEPE) common with dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) belongs to a group of proteins known as SIBLINGS proteins. This group of NCPs as a component of the extracellular matrix and dentin is responsible of forming small integrin-binding ligand, N-linked glycoprotein (**Malaval *et al.*, 2008; Staines *et al.*, 2012**).

One of the major NCPs found in mineralized tissue is bone sialoprotein. The function of this acid glycoprotein is not fully understood. It appears to mediate adhesion between cellular surfaces and extracellular matrix components and stimulates hydroxyapatite formation in vitro. (**Karmatschek *et al.*, 1997; Yang *et al*, 1995**). Secreted phosphoprotein-1 (SPP1), known as osteopontin, modulates both bone formation and resorption (**Standal *et al.*, 2004**). Through binding with vitronectin receptor, anchoring of osteoclasts are formed to the bone remodeling matrix, where the process is related with bone formation (**Chen *et al.*, 2014**). Fibronectin (FN1) is one of the first proteins produced by osteoblasts. It is a minor component of the bone matrix and directs the initial deposition of collagen fibrils. The presence of fibronectin is necessary to maintain the integrity of the collagen matrix (**Boskey, 2013**). By binding to other matrix proteins, it indirectly regulates mineralization and modifies their activities and regulates osteoblasts proliferation, differentiation and survival (**Sroga and Vashishth, 2012**). Matrix extracellular phosphoglycoprotein (MEPE) is protein, that are crucial for local matrix mineralization (**Mäkitie *et al.*, 2019**). Its function in bone mineralization is confirmed by the fact that its expression is increased during osteoblast matrix mineralization. In the case of bone, MEPE is primarily expressed by osteocytes (**Staines *et al*, 2012**). Decorin belongs to a family of small leucine-rich proteoglycans (SLRPs) and is a key regulator of collagen fibril and matrix assembly (**Robinson *et al.*, 2017**). Thrombospondin 2 (THBS2) belongs to the thrombospondin family and mediates cell-to-cell and cell-to-matrix interactions. Increase cortical bone density, acceleration of fracture healing and alters the pattern of load-induced bone formation are results of disrupted thrombospondin 2 expression. THBS2 participates in modulation the proliferation of osteoprogenitor cells and bone remodeling (**Gao *et al.*, 2017**). For proper osteoclast function and differentiation is crucial the precise regulation of Ca^{2+} dynamics. (**Kim *et al.*, 2012**). The plasma membrane calcium pump (PMCA) plays an important role in cellular calcium homeostasis (**Ryan *et al.*, 2015**). The *ATP2B1* gene, in hens located on chromosome 1, region 43 273 706–43 305 815 bp, encodes plasma membrane calcium ATPase 1 (PMCA1) (**Horecka *et al.*, 2015; Long *et al.*, 2017, Horecka *et al.*, 2018**).

Because most of studying proteins are involved in osteogenesis, bone remodelling and process of calcium metabolism, so polymorphisms of genes, encoding selected proteins, may play an important role in the pathogenesis of osteoporosis, by influencing protein formation and consequently bone formation. The aim of this work was to find out polymorphisms of selected genes and to identify possible associations with mechanical bone parameters that could be results of bone tissue disorders in laying hens.

MATERIAL AND METHODS

Samples of isolated DNA from ISA Brown hens were tested. In non-collagenous proteins encoding genes, 19 samples of each gene were sequenced in the pilot study. Blood samples were taken from hens, kept in enriched cage technology according to 74/99/EC Council Directive. The hens were fed with a balanced layer feed (116 to 170 g.hen^{-1}.day^{-1}) that contained: 875 g kg^{-1} dry matter (energy content MEN 11.1 MJ kg^{-1}); crude protein (170.7 g kg^{-1}); Ca (35.9 g kg^{-1}); and P (6.3 g kg^{-1}). A constant light-dark cycle (15:9, switching on at 04.00 h and switching off at 19.00 h) was maintained as recommended in technological instructions for ISA Brown pullets. Hens were slaughtered at the average age of 26 weeks. Samples were taken immediately after slaughter by decapitation and blood was stabilized with heparin. Isolation of DNA was carried out from 100 µl blood and commercially available DNA Lego kit (Top-Bio, Prague, Czech Republic) was used. The isolation proceeded according to the manufacturer's protocol. The analyzed bone physiological parameters were bone breaking strength of bones, bone length, width and bone mass, when analyses were performed on the femur. Right thigh was separated from the body of all animals and the femur was extracted out. After that all of muscles were removed. Bone strength was analyzed by universal testing machine TIRATEST 27025 (*TIRA Maschinenbau GmbH*, Schalkau, Germany) by three-point bending test. Bone length and width was assessed using Vernier calipers. Bone length was determined as the longest distance between the end of the distal and proximal epiphysis of the femur. The bone width was determined as the greatest distance between the *facies cranialis* and *facies caudalis* at the fracture point.

IBSP, SPP1, FN1, MEPE, DCN, THBS2 and *ATP2B1* genes testing

For the PCR amplification specific oligonucleotide primers were designed (Table 1) using Oligo software v4.0 (Molecular Biology Insights, Inc., Colorado Springs, CO, USA) and cycling conditions were 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 57 °C for 30 s, 72 °C for 1 min, and then 10 min at 72 °C after the final cycle. PCR amplifications were performed using a ABI Veriti 96-Well thermocycler (Life Technologies, Applied Biosystems) in 10 µl reaction volume.
The quality of PCR amplification and verifying of the correct PCR amplicon size was detected on 2.5% agarose gel stained with GoodView at 120V for 30 min using TBE buffer and compared with weight marker 100 bp DNA Ladder (M100) (Thermo Fisher Scientific Inc., Waltham, USA). The PCR sequencing template was prepared by mixing: 0.2 µl of the prepared PCR template (or 0.5 µl with poor quality of PCR product), 0.5 µl Terminator mix, 1.75 µl Terminator 5X buffer, 7.39 µl DI H2O and 0.16 µl 10 µM forward or reverse primer. Conditions of cycle sequencing was 96 °C for 1 min followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min.
For purification was performed using BigDye XTerminator Purification kit (Life Technologies, Applied Biosystems) adding 5 µl XTermination solution and 22.5 µl Sam solution to each PCR sample, reaching a final volume of 37.5 µl. The samples were vortexed for 30 min and centrifuged at 3200 rpm for 2 min. The supernatant liquid was removed from each sample and transferred to a plate prior to being processed by an ABI PRISM 3500 DNA analyzer (Life Technologies, Applied Biosystems). Sequence alignments were performed using SeqScape v2.7 (Life Technologies, Applied Biosystems).

Table 1 *IBSP, SPP1, FN1, MEPE, DCN, THBS2* primers

Gene	Primer sequence	Product size (bp)
IBSP	**Forward:** 5'-AGAGGAGCAGGATGTCAGTGT-3'	491
	Reverse: 5'-CTTGTTGCTTTATTGCGTTTC-3'	
SPP1	**Forward:** 5'-TTTCTTTGCTTGTGCTTTATCA-3'	598
	Reverse: 5'-TCAAGCCCTCAATCCTAAATC-3'	
FN1	**Forward:** 5'-CTTGGACTTGCTGGTGCTGTA-3'	676
	Reverse: 5'-GGTTTGTCTGTTGCCATTGC-3'	
MEPE	**Forward:** 5'-GAGCAGGATGGGGGCACT-3'	673
	Reverse: 5'-CCCCCGTGTGATGGTGAC-3'	
DCN	**Forward:** 5'-TGTGGCTTATTGTGTTGATTGTT-3'	567
	Reverse: 5'-ATGAACACACTCCTGGGCTTA -3'	
THBS2	**Forward:** 5'-TTTATCCTTTCAGCCACCCT-3'	320
	Reverse: 5'-ATTCTGCTGTTCTCTGCTTTCA-3'	

Legend: *IBSP* – integrin-binding sialoprotein, *SPP1* – osteopontin, *FN1* – fibronectin, *MEPE* – extracellular phosphoglycoprotein matrix, *DCN* – decorin, *THBS2* – thrombospondin 2

Statistical evaluation

Obtained data were analyzed using Kruskal-Wallis test with genotype as an independent variable and bone breaking strength, bone length, width and bone mass as dependent variables. All statistical analyses were performed by STATISTICA 12 statistical software (StatSoft Inc., Tulsa, USA). The overall level of statistical significance was defined as $P<0.05$.

RESULTS

Nineteen samples of each gene, which encoding non-collagenous proteins, were tested. Only one synonymous SNP (c.158C>T) polymorphism were discovered in the *DCN* gene. *MEPE* and *IBSP* genes showed non-specific amplification. Therefore, these genes were excluded from the analysis. Another of the tested gene, the *THBS2*, was monomorphic.

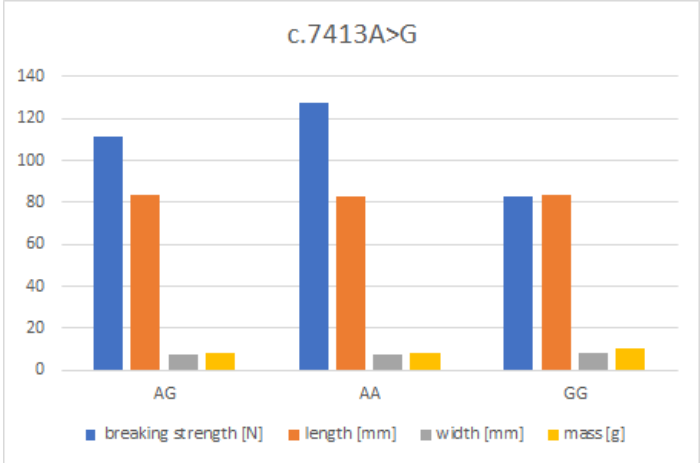

Figure 1 Effect of c.7413A>G polymorphism *FN1* gene on femur parameters

In the case of the *FN1* gene, a total of 4 polymorphisms were detected, three polymorphisms were found in the intron (c.7440+57G>A, c.7440+105G>A, c.7441-71T>C) and one in the exon (c.7413A>G), but this SNP was synonymous (not causing a change in the amino acid).
The allele frequencies of polymorphism in the *FN1* gene for c.7440+105G>A polymorphism were 0.10 for allele *A* and 0.90 for *G* allele with frequencies of the genotypes: 0.21 for *AG* genotype, 0.79 for *GG* genotype and *AA* genotype were not found. The allele frequencies of polymorphism for c.7440+57G>A, were 0.45 for allele *A* and 0.55 for allele *G*. Frequencies of the genotypes were 0.11 for *AA* genotype, 0.68 for *AG* genotype and 0.21 for *GG* genotype. Polymorphism of c.7441-71T>C showed allele frequencies: 0.58 for *T* allele and 0.42 for *C* allele, frequencies of genotypes were 0.22 for *TT* genotype, 0.72 for *TC* genotype and 0.06 for *CC* genotype.

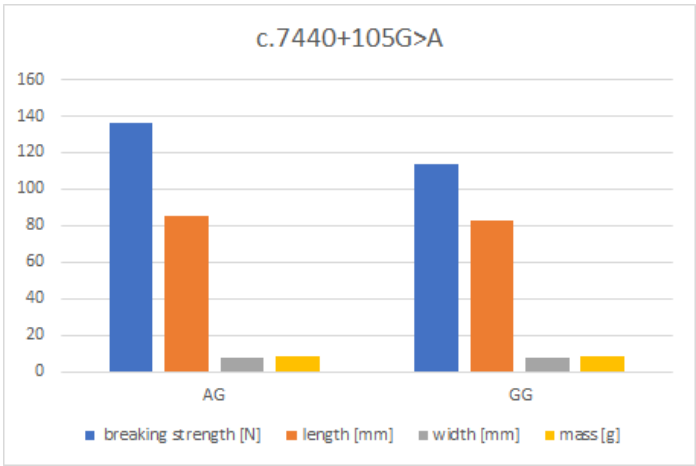

Figure 2 Effect of c.7440+105G>A polymorphism of *FN1* gene on femur parameters

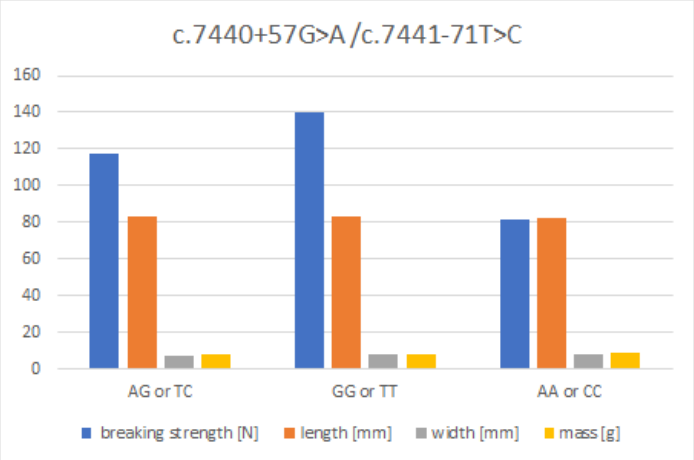

Figure 3 Effect of c.7440+57G>A/c.7441-71T>C polymorphisms of *FN1* gene on femur parameters

A total of 2 polymorphisms in the intron (c.362+117G>C, c.363-88G>C) were found in the selected section of the *SPP1* gene. In the *SPP1* gene, two intronic polymorphisms were found. The allele frequencies of c.362+117G>C were 0.19 for *C* allele and 0.81 for *G* allele, with genotype frequencies: 0.37 for *GC* genotype, 0.63 for *GG* genotype. *CC* genotype were not found. Similar result showed polymorphism c.363-88G>C, where allele frequencies were 0.81 for *C* allele and 0.19 for *G* allele, with genotype frequencies: 0.63 for *CC* genotype and 0.37 for *GC* genotype. *GG* genotype was not found.

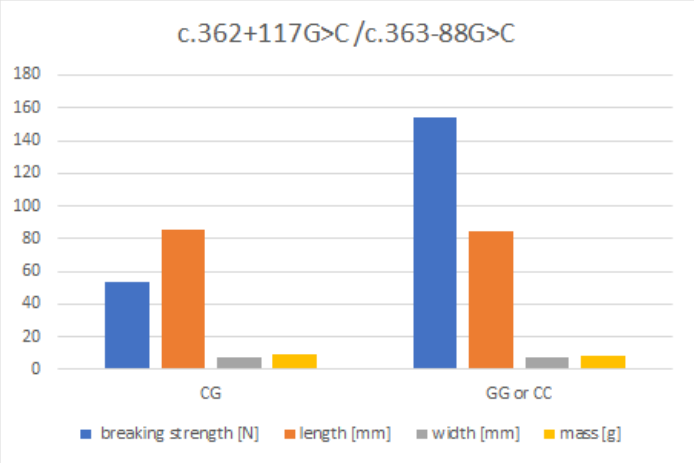

Figure 4 Effect of c.362+117G>C/c.363-88G>C polymorphisms of *SPP1* gene on femur parameters

The femur breaking strength had minimum and maximum values of 39.47 and 271.67 N respectively. Range of absolute values for bone length was between 79.24 and 89.41 mm, bone width 6.93 and 8,82 mm, bone mass 6.86 and 9.45 g. Despite some visible differences in bone parameters (Figure 1-4), the effects of polymorphisms were not proved.

DISCUSSION

This study was focused on finding polymorphisms that could be associate with bone parameters in laying hens, with a greater focus on non-collagenous proteins. There is no similar study focusing exclusively on non-collagenous proteins and very few studies to address gene polymorphisms associated with osteoporosis in laying hens. There are several studies on candidate genes and SNPs that are associated with osteoporosis in humans **(Dastgheib *et al*., 2016; Liu *et al*., 2017; Qin *et al*., 2016)**.

Guo *et al.* (2017) conducted a genome association study (GWAS). This study included 1534 laying hens and examining the quality of their bones. They mapped the greatest heritability for bone mineral density on chromosome 1, with a region of 165-171 Mb on GGA1 having a significant effect on bone quality. According to the NCBI genomic biology database, 4 genes (RANK, SERPINE3, INTS6 and POSTN) have been identified in this region and these genes might have an important effect on bone quality. They found nine SNPs that were associated with bone quality, and three of these genes (*RANKL*, *ADAMTS* and *SOST*) are known to be associated with human osteoporosis. This makes them suitable candidate genes for osteoporosis in laying hens. **Johnsson *et al.* (2015)** have identified several candidate genes affecting bone allocation and metabolism, which can also be used as a model for osteoporosis. They include in study a gene encoding a non-collagenous protein, osteonectin (SPARC). It contributes to mineralization by binding mineral crystals and it functions in bone remodeling. SPARC is a possible candidate gene and a gene that can affect both total bone and modular tissue. **Fornari *et al.* (2012)** deal with one selected polymorphism (A211G). This polymorphism is found in the bone sialoprotein gene and its association with the skeletal structure in a paternal line of broilers has been investigated. Association analysis showed several significant indicators, including the tibia width indicator, where the analysis was highly significant. **Raymond *et al.* (2018)** conducted an association study for bone strength in laying hens. Testing was performed on 752 laying hens belonging to the same population of pure lines. These laying hens were genotyped for a total of 580,961 SNPs, with 232,021 SNPs remaining after quality control. Associations with tibial breaking strength were tested for each SNP. A total of 52 SNPs were found that were significantly associated with the tibial breaking strength across chromosomes 1, 3, 8 and 16. Also, 5 distinct and novel QTLs on these chromosomes were identified, with the strongest association being detected in the QTL region at chromosome 8. Several candidate genes, including the BRD2 gene, were detected in these QTL regions. This gene is required for normal bone physiology.

Most of the detected SNPs in genes encoding non-collagenous proteins were found in the non-coding regions of the genes, introns. Since introns belong to the non-coding region of DNA, the polymorphisms found in this region may not seem to have the corresponding value.

However, intronic SNPs may potentially affect mRNA splicing. If the mRNA from a given gene can undergo alternative splicing, then this intron may be included in an alternative form of that protein, thereby affecting the expression of that protein and subsequently leading to abnormalities in the respective phenotype **(Cai *et al*., 2015; Cooper, 2010)**.

Most of the reported mutations are located in exonic sequences, although that >90 % of the gene sequences contain introns. However, the number of new pathogenic variants increases. The mutations that affect splicing disrupt highly conserved donor and acceptor sites at the exon-intron junctions, the branch-point sequence, and the polypyrimidine tract with various consequences. These consequences include exon skipping and the activation of cryptic splice sites. Recently, attention has been focused on mutations deep in intronic sequences, which affect exonic and intronic splicing enhancers or silencers. These less well-conserved auxiliary splicing sequences help to recognize and bind specific splicing regulatory proteins **(Seo *et al*., 2013)**. Therefore, SNPs not only in exons can play an important role in identifying specific associations.

CONCLUSION

Genetic factors are the basis for variability in bird susceptibility to osteoporosis and bone fractures. In laying hen breeds, skeletal problems are becoming an increasingly common problem, and it is known, that this problem is not only confined to conventional battery cages. If there is no reassessment of the approach to breeding laying hens, the problem of skeletal damage could be aggravated, especially by increasing production pressure. Studies of markers that could affect bone parameters, especially their strength, can contribute to both welfare and economic aspects of production. Therefore, this work tried to focus on the search for SNPs that could affect the monitored bone indicators. In genes encoding non-collagenous proteins there was no polymorphism that affected bone parameters in animals of the present study. Therefore, for some genes, it would be advisable to extend the study to other regions of the genes that would be explored, and methods optimized to allow further analysis. Also, the study could be extended to include new genes that are mentioned as candidates by the authors.

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