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ANALYSIS OF GENE EXPRESSION IN RABBIT MUSCLE

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ABSTRACT

Increasing consumer knowledge of the link between diet and health has raised the demand for high quality food. Meat and meat products may be considered as irreplaceable in human nutrition. Breeding livestock to higher content of lean meat and the use of modern hybrids entails problems with the quality of meat. Analysing of livestock genomes could get us a great deal of important information, which may significantly affect the improvement process. Domestic animals are invaluable resources for study of the molecular architecture of complex traits. Although the mapping of quantitative trait loci (QTL) responsible for economically important traits in domestic animals has achieved remarkable results in recent decades, not all of the genetic variation in the complex traits has been captured because of the low density of markers used in QTL mapping studies. The genome wide association study (GWAS), which utilizes high-density single-nucleotide polymorphism (SNP), provides a new way to tackle this issue. New technologies now allow producing microarrays containing thousands of hybridization probes on a single membrane or other solid support. We used microarray analysis to study gene expression in rabbit muscle during different developmental age stages. The outputs from GeneSpring GX sotware are presented in this work. After the evaluation of gene expression in rabbits, will be selected genes of interest in relation to meat quality parameters and will be further analyzed by the available methods of molecular biology and genetics.

Keywords: rabbit meat**;** meat quality**;** microarray; gene expression; candidate gene

INTRODUCTION

Increasing consumer knowledge of the link between diet and health has raised the awareness and demand for high quality food. Meat and meat products may be considered as irreplaceable in human nutrition, because they are important sources of protein, fats, essential amino acids, minerals (iron, selenium), vitamins (A, B12 and folic acid) and other nutrients **(Biesalski, 2005)**. Meat quality is characterized by a summary of all the factors which guarantee the nutritional, sensoric, hygienic and technological parameters of meat. Breeding livestock to higher content of lean meat and the use of modern hybrids also entails problems with the quality of meat. Consequently we encounter with the occurrence of defective physico-chemical, technological and sensory parameters of meat. Efforts of geneticists, breeders and processors are therefore now more directed at improving the meat quality and meat products **(Brestenský et al., 2002)**. Analysing of livestock genomes could get us a great deal of important information, which may significantly affect the improvement process. For example, mapping the genomes of cattle revealed a number of genes responsible for influencing the quality and quantity of meat **(Gábor et al., 2012)**. Knowledge of the sequence of the entire genome can immediately start studying the expression of any gene in the organism. Nowadays it is possible to synthesized oligonucleotide hybridization probes complementary to a transcript segment of any gene and we can use PCR method to produce a million copies of each gene in the genome. It also allows monitoring changes in gene expression of the entire genome in time during development, or in response to some changes in the environment. New technologies now allow producing microarrays containing thousands of hybridization probes on a single membrane or other solid support. Gene chips can carry more than 10000 oligonucleotide probes on a silicon membrane size of a few square centimeters **(Snustad & Simmons, 2009)**. Using conventional methods of molecular biology and genetics and the application of the latest technologies in the food industry, including microarray, it is possible to obtain accurate results with a specific meaningful values at a much shorter time.

Domestic animals are invaluable resources for study of the molecular architecture of complex traits. Although the mapping of quantitative trait loci (QTL) responsible for economically important traits in domestic animals has achieved remarkable results in recent decades, not all of the genetic variation in the complex traits has been captured because of the low density of markers used in QTL mapping studies. The genome wide association study (GWAS), which utilizes high-density single-nucleotide polymorphism (SNP), provides a new way to tackle this issue **(Zhang et al., 2012a)**. These studies help scientists widely to reveal the connection between individual SNPs and disorders that are passed down from one generation to another according to Mendel's laws of heredity **(Norrgard, 2008)**. Depending on the position and flanking sequences in the gene, SNPs may have different functional effects on protein sequence, transcriptional regulation, RNA splicing or miRNA binding. Decades various ongoing studies provide a wealth of information about

genes, chromosomal regions and pathways that may be associated with certain types of disorders and diseases. GWAS offer an agnostic approach to research associations between SNPs and disorders, and the results of these studies offer a countless data for studies of new generation **(Xu & Taylor, 2009)**. Currently, there are many information obtained through GWAS for a lot of livestock. Found information for well-studied species, can be used for comparison with other less explored.

Sporer, et al., 2011 developed and validated a turkey skeletal muscle-specific microarray as a tool for functional genomics studies and identified gene pathways and uncovered novel genes important in turkey muscle growth and development. In future experiments they will focus further on several of these candidate genes and the expression and mechanism of action of their protein products. Skeletal muscle growth and development from embryo to adult consists of a series of carefully regulated changes in gene expression. Understanding these developmental changes in agriculturally important species is essential to the production of high quality meat products. For example, consumer demand for lean, inexpensive meat products has driven the turkey industry to unprecedented production through intensive genetic selection. However, achievements of increased body weight and muscle mass have been countered by an increased incidence of myopathies and meat quality defects. The goals of study were to utilize this microarray to elucidate functional pathways of genes responsible for key events in turkey skeletal muscle development and to compare differences in gene expression between two genetic lines of turkeys. A total of 3474 genes were differentially expressed between at least two developmental stages across both genetic lines. Of these, 2544 genes were significantly affected for the RBC2 birds, whereas 2248 were significantly affected for the F birds. A greater number of genes were up-regulated in the earlier developmental stage of direct comparisons as compared to those that were down-regulated, and this observation was more pronounced in the RBC2 line than the F line.

Rabbit (*Oryctolagus cuniculus*) is very significant and globally widespread species of animal used as a laboratory model for research anatomy and physiology of the human diseases. It also serves as a model for studies of larger production domesticated species. Moreover, it is in Central Europe and certain parts of Asia reared as a meat animal. Beneficial properties of rabbit meat are widely recognized and have recently been examined thoroughly **(Paredi et al., 2012)**.

The main area of livestock research deals with the study of muscle fibers. MSTN and MYOG are considered candidate genes in relation to the growth and development of muscles. Myostatin (MSTN) is a secreted growth factor that is expressed predominantly in skeletal muscle, which negatively regulates the growth of skeletal muscle, whereas myogenin (MYOG) is involved in many important processes such as differentiation of myofibrillar cells. Therefore, it is expected that these genes may affect the growth of rabbit muscle. So far, however, their impact has not been clearly confirmed **(Kuang et al., 2012)**. Important endocrine factor growth hormone (GH) regulates the metabolism of growth and development. During the biological process, the first step is binding the GH to growth hormone receptor (GHR), followed by activation of the JAK-STAT pathway and expression of IGF1 (Insulin-like growth factor 1) and the other target genes. In addition, the GHR is a member of the superfamily of cytokine / hematopoietin receptors and consists of three functional domains, extracellular (ligand-binding) domain, a transmembrane domain and cytoplasmic domain (signal-transferring). GH binding to a receptor causes GHR dimerization and initiates signaling cascades through the cytoplasmic domain. GHR gene polymorphisms may affect the binding capacity of GH, which is considered as a candidate gene affecting growth and development carcasses in livestock. In rabbits, however, has not yet been extensively studied **(Zhang et al., 2012b)**. Among the candidate molecules involved in the regulation of energy homeostasis gets a special interest the melanocortin 4 receptor (MC4R), it is doubled G-protein receptor. Not only disruption of the gene encoding this receptor results in obesity in mice, but the interaction between leptin and MC4R signaling pathways highlights the correlation between body weight and food intake. Moreover, the mutations in the MC4R gene in swine associated with growth and fat thickness in relation to the feed intake. The cDNA encoding bovine melanocortin 4 receptor (MC4R) has been cloned and sequenced. Comparing with human, porcine and rat homologues showed 87, 85 and 89% identity at the DNA level, respectively more than 90% identity at the protein level **(Haegeman et al., 2001)**. In different breeds of rabbits were found SNPs in the coding region of MC4R gene. At position 237 bp were exchanged adenine for guanine. Allele A prevailed over the G allele in all studied meat breed. Analysis for determination the effect of genotype on rabbit utility confirmed the significant relationship between genotype AG, body weight and feed conversion. The results showed that the MC4R gene could be a candidate gene for the mass and slaughter yield in rabbits **(Jianq et al., 2008)b**.

MATERIAL AND METHODOLOGY

Analyzed animals were rabbits (males) inbred line M91, aged 6-weeks followed by 2, 3, and 5 months, in order to study the variation during ontogenetic developement. Breeding was carried out under the statute and zootechnical conditions of the experimental breeding rabbits at ÚMHZ CVŽV Nitra. The experiment was carried out 3 times.

Samples of muscle tissue from hind leg were collected immediately after slaughter of the animal using a sterile scalpel and under strict sterile conditions were immediately placed into pre-chilled tubes containing an equivalent amount of a stabilizing solution of RNA in tissue RNA Later ™ (Sigma).

RNA isolation:

Samples of muscle tissue were transferred to new plastic tubes in RNase-free conditions and immediately frozen in liquid nitrogen for 30 minutes. Then rapidly removed and homogenized in a small amount of liquid nitrogen in sterile friction plates using a mortar. About 30 mg of the homogenate was used for the isolation of RNA using a commercial kit Gene Jet™ RNA Purification Kit

(Fermentas) as described in the manual of the relevant kit. The concentration and quality of isolated RNA was measured by UV-VIS spectrophotometer NanoDrop ™.

RNA amplification and labeling:

Pure RNA was then amplified and labeled using the Two-Color RNA Spike-In Kit (Agilent) as described in the kit manual. After labeling, the samples were purified by converting Clean up protocol of a commercial kit Gene Jet™ RNA Purification Kit (Fermentas). Subsequently, the samples were again measured on a NanoDrop™ using module for microarray. Using this module it is possible to determine the amount of dye (pmol) built on micrograms of DNA / RNA.

Hybridization:

Labeled samples were applied to the Rabbit Gene Expression Microarray chip (Agilent), which were covered with a cover glass to separate the fields on the chip. Prepared chip was placed in a hybridization oven with carousel where the slide was revolving to ensure a constant flow of the sample through that field. Hybridization was carried out for 17 hours at 65 °C. The next step included washing in Wash solutions to remove unhybridized cRNA.

Scanning the microarray chip and data processing: Microarray slides were scanned using microarray scanner (Agilent) with two lasers: SHG-YAG laser (532 nm) and helium-neon laser (633 nm) at a resolution of 10 μm. TIFF images were visualized and processed using Feature Extraction Software 10.7.3.1 and text files were further analyzed by GeneSpring GX software.

RESULTS AND DISCUSSION

In addition to analyzing the different stages alone were also carried out two-color comparative experiments whose outputs (from GeneSpring GX) are presented in this work. They were compared with each other age stages in the order of 1. (6 weeks) versus 2. (2 months), 2. (2 months) versus 3. (3 months), 3. (3 months) versus 5. (5 months) and 5. (5 months) versus 1. (6 weeks). The samples were always labeled as green versus red: Cy3 versus Cy5 plot (Raw). Expression value is in this case referenced to a red sample.

One of the next steps in evaluating gene expression tends to be inclusion of genes into metabolic pathways and analysis of gene ontology. GeneSpring GX software provides these options but not for the rabbit genome. Therefore are included in this work only comparative analyzes, which allow easier manipulation with such a large amount of data (an average of 10000 genes). Summary of resulting data is shown in table 1. After evaluating the differences in expression between stages were selected only genes with expression changes greater than 2.

Comparison of gene expression in 1. and 2. age stage

In the analysis of differential gene expression in muscle tissue at 1. age stage (six weeks) and 2. age stage (2 months) were 7870 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 960 and down regulated genes with significant (multiple) expression changes were 635.

Comparison of gene expression in 2. and 3. age stage

In the analysis of differential gene expression in muscle tissue at 2. age stage (2 months) and 3. age stage (3 months) were 13802 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 380 and down regulated genes with significant (multiple) expression changes were 679.

Comparison of gene expression in 3. and 5. age stage

In the analysis of differential gene expression in muscle tissue at 3. age stage (3 months) and 5. age stage (5 months) were 8993 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 685 and down regulated genes with significant (multiple) expression changes were 1338.

Comparison of gene expression in 5. and 1. age stage

In the analysis of differential gene expression in muscle tissue at 5. age stage (5 months) and 1. age stage (6 weeks) were 9921 genes with expression changes greater than 2. Up regulated genes with significant (multiple) expression changes were 861 and down regulated genes with significant (multiple) expression changes were 988.

Table 1 Overview of gene expression in different age stages.

Age stage	Genes with expression changes >2 (total)	Significantly up regulated genes	Significantly down regulated genes
1. versus 2.	7870	960	635
2. versus 3.	13 802	380	679
3. versus 5.	8993	685	1338
5. versus 1.	9921	861	988

CONCLUSION

Based on these results we determine the application outputs focused on quality and safety of rabbit meat. We have found that there has been a change in gene expression in rabbit muscle, depending on their age. Genes have been identified, and further study will be analyzed individually using real time PCR. We are expecting to receive comprehensive information about gene expression in muscle cells of rabbits during critical stages of ontogenesis of muscle tissue. After the evaluation of gene expression in rabbits, will be selected genes of interest in relation to meat quality parameters and will be further analyzed by the available methods of molecular biology and genetics. Evaluating the gene expression could help us obtain knowledge and information about the energy metabolism of myocytes, which could then be used to determine the significant associations to the rabbit meat quality parameters.

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