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Microarray analyses to identify differentially expressed genes for assessing meat quality in swine

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ABSTRACT: In order to identify candidate genes and molecular mechanisms that influence meat quality and production in pigs, microarray experiments were carried out to find differences in gene expression levels between two pools of six individuals, constituting the extreme tails of the Gaussian distribution of seven adjusted phenotypes of 100 Landrace and Large White animals. The phenotypes considered in this study were: muscle compactness, marbling, colour uniformity, fat covering, colour, dorsal fat, thickness, ham fat thickness. 437 differentially expressed ESTs (Expressed Sequence Tags) were found, clustering in different pathways according to their ontology. In particular, 73 functional categories were identified and ten of them could have a role in meat quality. Among the ESTs belonging to these pathways, seven of them were selected to be validated in quantitative real-time RT-PCR.

Key words: Meat quality, Microarray, Candidate genes.

INTRODUCTION - Genes expressed in skeletal muscle could be candidate genes for meat quality. They play a central role in metabolism, development and physiology of this tissue. Gene expression profiling in the skeletal muscle had already been investigated by different molecular tools, such as microarrays (Bai *et al*. 2003), the Suppressive Subtractive Hybridisation (SSH) (Gorni *et al*., 2006) and cDNA libraries (Davoli *et al*. 2002). Microarray profiling can be very helpful to discover molecular processes that govern the phenotypic characteristics of porcine skeletal muscles and expression of specific fibre types. This information is important for enhancing the efficiency of livestock production (Bai *et al*. 2003). Microarray technologies for transcriptional profiling allow to extract genome-wide expression data, and numerous methods have been developed to analyse this type of data, including clustering procedures that divide genes into disjoint or overlapping sets representing all or part of the genes being analysed.

In this study microarrays were used to identify any difference in gene expression levels between two pools of six individuals, constituting the extreme tails of the Gaussian distribution of seven adjusted phenotypes of 100 Landrace and Large White animals. The aim of the study is to identify candidate genes and molecular mechanisms that influence pork meat quality and production.

MATERIAL AND METHODS - Tissue samples were collected from skeletal muscles of 100 individuals of Landrace and Large White animals. Samples were collected immediately after animals slaughtering and stored in RNA later (Sigma-Aldrich). Total RNA was isolated from the skeletal muscle (*longissimus dorsi*) of 12 of these animals (6 of each extreme tail of the Gaussian distribution of seven adjusted phenotypes) using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. RNA purity, integrity and concentrations were evaluated on the Agilent 2100 Bioanalyzer.

Total RNA samples from the six animals of each extreme tail were pooled to give a total amount of 5 µg. RNA labelled probes were obtained using Amino Allyl MessageAmp-kit (Ambion) and were hybridised onto microarray

Table 1. Description of the 13 differentially expressed ESTs found in the functional categories. The seven sequences underlined were selected to be validated in real-time RT-PCR.

Human_Ref Seq	Gene Simbol	Description	Cytoband	Pathway
NM_001517	GTF2H4	general transcription factor IIH	6p21.3	Transcription, Basal transcription factors
NM_007055	POLR3A	polymerase (rna) III	10q22-q23	Transcription
NM_005643	TAF11	taf11 rna polymerase II, tata box binding protein (tbp)	6p21.31	Transcription,Basal transcription factors
NM_003194	TBP	tata box binding protein	6q27	Transcription,Basal transcription factors
NM_002194	INPP1	inositol polyphosphate-1-phosphatase	2q32	Phosphatidylinositol signaling system, Signal Transduction
NM_005027	PIK3R2	phosphoinositide-3-kinase, regulatory subunit 2	19q13.2-q13.4	Phosphatidylinositol signaling system, Signal Transduction
NM_130393	PTPRD	protein tyrosine phosphatase	19p13.3, 9p23-p24.3	Phosphatidylinositol signaling system, Signal Transduction
NM_182811	PLCG1	phospholipase c, gamma 1	-	Phosphatidylinositol sig naling system, Signal Transduction
NM_003805	CRADD	casp2 and ripk1 domain containing	12q21.33-q23.1	Apoptosis, Cell Growth and Death
NM_001605	AARS	alanyl-trna synthetase	16q22	Translation
NM_000076	CDKN1C	cyclin-dependent kinase inhibitor 1c	11p15.5	Cell cycle, Cell Growth and Death
NM_170692	RASAL2	ras protein activator like 2	1q24	Signal Transduction, MAPKsignaling path
NM_001749	CAPNS1	calpain, small subunit 1	19q13.12	Cell Communication

slides spotted in single replicate with the 70mer Pig Genome Oligo Set Version 1.0 representing the 10,665 *Sus scrofa* gene sequences (Qiagen) using the Sigma hybridization chambers at 42°C for 48 hours. Six independent hybridization experiments were carried out with three replicates for each microarray experiment, including dye-swaps. Fluorescent signals were measured with a ScanArray Lite laser scanning system (PerkinElmer). Spot analysis of 10-µm-resolution TIFF images was carried out by using the Scan-Array Express Microarray Analysis System 2.1 software supplied with the scanner.

Lowess normalization is performed by the Scan-Array Express Microarray Analysis System 2.1 software. The success of the normalization was tested by visualization of the M-A plots.

For identifying differentially expressed ESTs, a statistical analysis based on the paired t-test between the upper and lower tail pools was performed. The dependent variables were the log 2 transformed intensity values. To evaluate significance, these t-values were compared against a critical value of 0.05, considering the Bonferroni correction for multiple tests (N = 10,665).

437 differentially expressed ESTs were found, ordered for ascending p-value and corrected for false discovery rate. To classify the differentially expressed ESTs into functional pathways, a computerized analysis approach using Gene Ontology (GO) (http://www.geneontology.org) was performed. Data mining was performed using the software

EASE (Expression Analysis Systematic Explorer), available in http://www.DAVID.niaid.nih.gov, (Glynn *et al*., 2003). To validate microarray results, quantitative real-time RT-PCR was performed using an ABI 5700 instrument, using the AppliedBiosystems SYBR-Green® fluorophore on seven selected porcine genes: INPP1, PIK3R2, PTPRD, PLCG1, CRADD, CDKN1C, CAPNS1 (Table 1). The protocol, based on the use of the relative standard curve, was performed as previously described by Gorni *et al*., 2006. Three different housekeeping genes (b-actin, rRNA 18S and ribosomial protein L35) were chosen for the normalization of the samples. The Relative Standard Method (Pfaffl, 2001) was applied to investigate the differential expression of the ESTs. Data from the RT-PCR were analyzed using the *t*-test. Results were considered to be significant for p-value < 0.05.
To evaluate the differences in gene expression levels, microarray experiments were performed using pooled RNA extracted from porcine skeletal muscles of 12 animals, belonging to the extreme tails of the Gaussian distribution of seven adjusted phenotypes. The phenotypes considered were: muscle compactness, marbling, colour uniformity, fat covering, colour, dorsal fat, thickness, ham fat thickness. Three replicates of each microarray experiment were carried out, each one with dye-swap.

RESULTS AND CONCLUSIONS - After the normalization, statistical analysis was performed as described in Matherials and Method. 437 differentially expressed ESTs were found, ordered for ascending p-value, corrected for false discovery rate and classified into functional pathways according to GO classification derived from EASE analysis. The interpretation of our microarray results was not trivial because of a lack of information about the swine genes. So, the swine differentially expressed ESTs were analyzed based on homology comparison with human genes. 73 categories in the biochemical pathway were identified. Ten of them supported the other 63 pathways and they could have a role in meat quality. Table 1 describes all the 13 differentially expressed ESTs belonging to these functional categories. Among these sequences, we selected seven of them to be validated in quantitative real-time RT-PCR.
Our results demonstrate the power of microarray analysis in identifying candidate genes that influence swine meat quality. The challenge will be confirming these associations and demonstrating how these genes are involved in the relevant muscle phenotypes.

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