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Microarray analysis of gene expression profiles of pig muscle in response to cold stress

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Min pigs, a major breed of the Heilongjiang Province in PR, are noted for their tolerance to cold. In severely cold weather (–20°C) they can stay in the open much longer without shivering or squealing than most other breeds. Selection for tolerance to environmental stress has traditionally been counterproductive in domestic animal production. Generally, as animals acclimatize to environmental stressors they reduce or divert metabolizable energy from production to balance heat gain and loss (Keister *et al.* 2002). Improved tools in molecular analysis of gene expression will permit improvement in the accuracy and intensity of use of genetic markers. There are many reports on use of chip technology research on the functional genes of animals (Lu *et al.* 2011), but similar study on pig's cold stress has not been reported. To further characterize the mechanisms and genes leading to cold stress, cDNA microarray analyses were developed to construct gene expression profile and screen differentially expressed genes in the muscle at different temperature. This study will be beneficial in elucidating the molecular mechanism of cold stress in chickens.

Min piglets (12), 75-day-old, procured from 3 different litters were randomly divided into the control group (C, n=6) and the cold-stressed group (E, n=6). The piglets of control were placed into the cages which was heated to 10±2°C and the piglets of experimental were placed into the cages, which was cooled to –20°C. Both groups were fed the same feed. After 13 days, piglets were killed and the leg muscles from different piglets were collected.

RNA extraction and microarray hybridization

Total RNA was isolated from approximately 0.5 cm^3 of tissue sample by homogenization with a polytron bench top homogenizer in 3 mL of TRIzol, and extracted according to the manufacturer's protocol. RNA integrity and concentrations were measured on a bioanalyzer and spectrophotometer respectively. All samples were of high quality with an RNA integrity number (RIN-value) between 7.6 and 8.7 and 260/280 ratios greater than 2.00. 500–ng total RNA per sample was used for microarray hybridization. Affymetrix GeneChip®, containing 24123 genes, of which 22458 genes are known genes, 1665 of other probes, 2000 functional class, 221 proteins, 135 chromosome classes, was used in this study.

Microarray data analysis

Microarray data were normalized using algorithms supplied with the feature extraction software. After data normalization, a final quality control filter was applied, whereby genes expressing excessive biological variability were discarded. Bioinformatics analyses, including

Table 1. Primer pairs used to analyze gene expression by quantitative RT-PCR

Gene	Primer sequence	Product size (bp)	GenBank accession number
CYP1A1	F:5'-AAGAGGATGGACGAGAATGCC -3'R:5'-GGTGAAGGGAACGAAGGAGGT -3'	282	NM_214412
FST	F:5'-AAACCTACCGCAACGAATGTG -3'R:5'-TGCCAGCCTTGAAATCCCATA -3'	381	NM_001003662
DDX58	F:5'-TTACCTTTGTCACCAGCACCA -3'R:5'- ACGAATCTGACCAGGAACCAT -3'	335	AF319661.1
ZNF12	F:5'-AAGGGAACCCTCACTGTCCATC -3'R:5'-GTCAGAGTAGACTTTCGGCAG -3'	200	XM_003125424
PDZRN3	F:5'-CGGGCCGTCCTCAAAGAATCTGC -3'R:5'-CATCTGGCTCTGTGCGTACTCG -3'	298	XM_003132326

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hierarchical clustering, GO terms (molecular function, biological processes, cellular components), and KEGG pathway analysis, were conducted using the SAS software program (http://sas. ebioservice.com/), which is based on both the GO database (http://www.geneontology.org/) and the KEGG database (http://www.genome.jp/kegg/pathway.html).

All results are presented as the mean±SD of 6 piglets per group. The differences between each group were analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0. A p-value of <0.05 was considered significant.

Real-time quantitative RT-PCR

The housekeeping gene, ß-actin, was run in all reactions separately under the same experimental conditions. Real-time PCR was performed as per Wong *et al*. (Wong *et al.* 2005).

Table 2. List of differential expression genes between 2 groups (fold change >2)

ID	Gene	Gene symbol	GenBank accession number	Location	Difference of means
Downregulated genes					
1	Ssc.2641.1	unknown	AW313822	unknown	–1.05
2	Ssc.29281.1	unknown	CO953055	unknown	–1.40
3	Ssc.30216.1	unknown	CO988299	unknown	–1.10
4	Ssc.30008.1	unknown	CO947798	unknown	–2.35
5	Ssc.30027.1	unknown	CO948050	unknown	–1.12
6	Ssc.10799.1	unknown	BQ598004	unknown	–1.02
7	Ssc.10285.1	unknown	BI400467	unknown	–1.46
8	Ssc.26005.1	unknown	BX926252	unknown	–1.55
9	Ssc.17718.1	unknown	BQ604042	unknown	–1.12
10	Ssc.26456.1	MCA-32	CN069543	unknown	–1.03
11	myxo-virus resistance 1	MX1	NM_214061	13	–1.25
12	2', 5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	NM_214303	14	–1.38
13	cytochrome P450 1A1	CYP1A1	NM_214412	7	–1.21
14	inflammatory response protein 6	IRG6	NM_213817.1	3	–1.45
15	interferon stimulated exonuclease gene 20kDa	ISG20	NM_001005351	unknown	–1.29
16	IgG heavy chain	LOC396781	NM_213828	unknown	–1.35
17	follistatin	FST	NM_001003662	16	–1.04
18	leukocyte antimicrobial peptide precursor	PG–2	L24745	unknown	–1.97
19	Ig gamma 2b chain constant region/ IgG heavy chain	IGG2B/ LOC396781	BX670708	unknown	–1.04
20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	DDX58	AF319661.1	unknown	–1.08
21	proline dehydrogenase (oxidase) 1	PRODH	AK231562	unknown	–1.33
22	brain expressed X-linked 1	BEX1	XM_003135280	X	–1.13
23	S100 calcium binding protein A9	S100A9	NM_001177906	4	–1.07
24	Interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1), transcript variant 2	IFIT1	XM_003133142	14	–1.22
25	ISG15 ubiquitin-like modifier	ISG15	NM_001128469	unknown	–1.26
26	chemokine (C-X-C motif) ligand 9	CXCL9	NM_001114289	8	–1.07
27	numb homolog (Drosophila), transcript variant 1	NUMB	XM_003128649	7	–1.09
Upregulated genes					
28	Ssc.7399.1	unknown	BF712467	unknown	1.01
29	Ssc.22211.2	unknown	CF794439	unknown	1.76
30	zinc finger protein 12	ZNF12	XM_003125424	3	1.04
31	chemokine (C-X-C motif) ligand 2	CXCL2	XM_003126160	5	1.91
32	haptocorrin	haptocorrin	X52566	unknown	1.47
33	PDZ domain containing ring finger 3	PDZRN3	XM_003132326	13	1.17
34	solute carrier family 23 member 3	SLC23A3	XM_001925526	15	1.57

The raw data from each experiment was used to determine the relative levels of expression for each gene by the delta-delta CT method as described by Hettinger *et al*. (Hettinger *et al.* 2001). The T-test is used to determine whether there is a significant difference between 2 group means. A P-value of <0.05 was chosen as the screening criterion of significantly different expression standard. The sequences of the primers used for gene expression and the sizes of their products are shown in Table 1.

Differential gene expression

This led to the identification of a total of 34 genes that were differentially expressed, of which 7 genes were significantly upregulated and 27 genes were significantly downregulated (Table 2).

Function prediction and analysis for differentially expressed genes

In this study, 7 IFN-related genes were down-regulated after cold-stress, such as OAS1, IRG6, ISG20, DDX58, IFITl, ISG15, CXCL9. We presume that cold stress may decrease the body's ability to anti-virus. The other expression levels were significantly decreased genes: MX1 is directly related to anti-virus; CYP1A1 and P450 are related to regulation of body temperature; FST is related to growth; BEX1 is related to seminiferous tubule development and spermatogenesis; S100A9; and Numb are related to cell proliferation, differentiation and apoptosis, one antimicrobial peptides of PG–2 and one mitochondrial enzyme of PRODH.

In 5 upregulated genes, except PDZRN3's function is unknown, others belong to different superfamily. Such as ZNF12 – it is a member of the krueppel C2H2-type zinc-finger protein family and encodes a protein with 8 C2H2-type zinc fingers and a KRAB domain. This nuclear protein is involved in developmental control of gene expression. Alternate transcriptional splice variants, encoding different isoforms, have been characterized (Danielle *et al.* 2006). CXCL2 is a small cytokine belonging to the CXC chemokine family. This chemokine is secteted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells (Wolpe *et al.* 1989).

Different gene clustering analysis of pathways

We used the database of KEGG to find the potential pathway, but did not find any pathway. Speculated that this may be due to the fact that the current pig genome annotation information, GO and Pathway annotation information are also limited. It led to a belief that GO and Pathway enrichment analysis is difficult to obtain biologically meaningful results. Hierarchical cluster analysis results showed that there were 3 clusterings– immune response clustering (OAS1, CXCL2, DDX58 and CXCL9), response to virus clustering (MX1, IRG6 and DDX58), and RNA binding clustering (OAS1, ISG20 and DDX58).

Validation of gene expression data by real-time quantitative PCR

To validate the microarray results, we performed real-time quantitative PCR for 5 genes. These genes were selected randomly. Fig.1 shows the comparison between the microarray and real-time RT-PCR results.

Stress response is one of the most universal reactions among biological organisms but is also a resistant defense process. Heat stress and cold stress are common stress

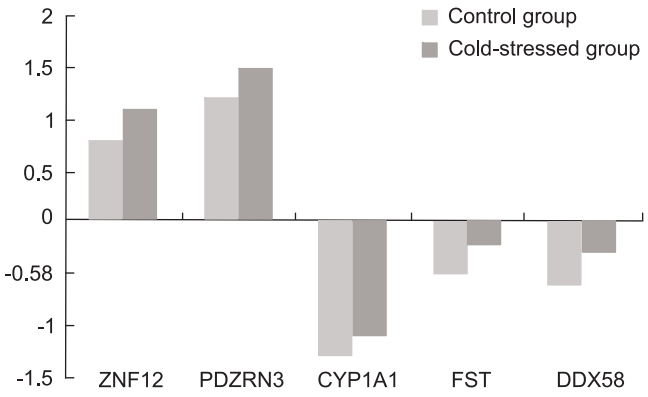

Fig.1. Application of real-time method for demonstrate pig expression chip.

conditions in domestic animal breeding. Four factors contribute to cold stress: cold temperatures, high or cold wind, dampness and cold water. A cold environment forces the body to work harder to maintain its temperature. Cold air, water, and snow all draw heat from the body. Cold temperatures increases the pig's energy requirements. Additional feed is required to maintain its temperature. Therefore, cold stress usually results in an increase in feed intake. In extreme conditions, feed intake can increase as much as 25%. Therefore, the study of the cold stress response in domestic animals is very important.

In this study, when piglets were exposed to cold stress for 13 days, 7 IFN-related genes were down-regulated significantly. This research proves that cold stress can increase the risk of illness and weaken the immune system. Sagher *et al.* (1975) found that cold stress could significantly decrease the body weight, breast and thigh muscle weight in chickens. In this study, we also found a functional gene (FST), which is significant correlation with the growth trait, was significantly decreased (Kota *et al.* 2009). In fact, in this research, we also found that the body weights of the normal piglets increased more than stressed piglets. May be we can say, cold stress can reduce the immune system's ability to fight infections and can affect growth.

SUMMARY

Ambient temperature is a critical factor that affects

biological organisms in many ways. In this study, the authors investigated gene expression changes in Min pig muscle in response to cold stress. Female Min pigs were randomly divided into control and cold-stressed groups. Control group was housed at 10±2°C; the cold-stressed group was housed at –20±3°C for 13 days. The results showed that 34 genes were differentially expressed, of which 7 genes were significantly upregulated and 27 genes were significantly downregulated. Subsequent bioinformatics analyses revealed that the differentially expressed genes were mainly related to immune response, response to virus, and RNA binding. The bioinformatics analysis of the differentially expressed genes should be beneficial to further investigations on the underlying mechanisms involved in cold stress–induced damage in the muscle.

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