USE OF BOVINE MICROARRAY ANALYSIS TO IDENTIFY NEW BIOLOGICAL PATHWAY FOR MARBLING IN HANWOO (KOREAN CATTLE)

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ABSTRACT

Marbling is a major trait in determining economic profit in the Korean beef industry. However, the underlying biology of muscles with divergent marbling phenotypes is still poorly understood in cattle. In this study, we attempted to detect differentially expressed genes in *M. longissimus dorsi* with divergent marbling scores using an Affymetrix bovine gene expression array. Three data-processing methods (MAS5.0, GCRMA and RMA) were implemented to test for differential expression (DE). A total of 21 differentially expressed genes were validated by real-time PCR. Both methods showed a concordance in the gene expression fold change between the data arising from microarray and real time PCR. Gene Ontology (GO) and pathway analysis demonstrated that several genes (*ADAMTS4, CYP51A* and *SQLE*) more highly expressed by high marbled muscle are involved in a protein catabolic process and a cholesterol biosynthesis process. In addition, pathway analysis revealed that *ADAMTS4* is activated by three regulators; *IL-17A, TNFα* and *TGFβ1*. QRT-PCR was used to investigate their gene expression in muscle with divergent IMF contents. The results demonstrate that *ADAMTS4* and *TGFβ1* are associated with increasing marbling fat. In conclusion, the *ADAMTS4/TGFβ1* pathway is involved in the phenotypic differences between high and low marbled groups.

I. INTRODUCTION

Intramuscular fat deposition within the musculature starts to become visible at the age of 12 months and the rate increases from 15 months to 24 months (Nishimura *et al.* 1999). The initial formation of visible intramuscular fat seems to be driven through the development of adipocytes (Pethick *et al.* 2006). Hocquette *et al.* (1998) reported that metabolic differences to balance triacylgriceride (TAG) storage within muscle such as fatty acid trafficking and oxidation of fatty acid in mitochondria are known as an important biological contributor in determining marbling levels in the later stages of finishing cattle.

Kokta *et al.* (2004) reviewed the interaction between myogenic cells and adipocytes to determine the rate and extent of myogenesis and adipogenesis during animal growth. Fat and muscle development are regulated by a number of complex biological pathways such as adenoreceptor signaling (Fruhbeck *et al*. 2001), cytokine signaling pathway (Shin *et al*. 2003) and a wide range of hormonal and transcriptional factors. As such, the partitioning between muscle and fat is a determinant of energy balance. Therefore, marbling differences might be expressed by metabolic differences resulting from a complex mechanism of communication among cells (Sorisky *et al*. 1999).

This study identified differentially expressed genes in *M. longissimus dorsi* of animals with divergent marbling phenotypes. We selected high and low marbling animals and then looked at between-group differential expression of genes using the bovine genome array (Affymetrix Inc, USA).

II. MATERIALS AND METHODS

Animal. From a group of 90 steers, the 5 highest and the 5 lowest were selected based on the marbling score. Carcass measurements and chemical fat percentage AOAC (1990) of the muscle sample were measured. Summary statistics for the animals and muscle samples used in this study are shown in Table 1.

Target preparation and high-density array hybridization. Double stranded cDNA was synthesized from 3 μg mRNA using a Genechip Expression 3'-Amplification One Cycle Synthesis kit (Affymetrix Inc. USA). After the cDNA was purified, Biotin-labeled cRNA was synthesized in vitro using the Gene chip Expression 3'-Amplification reagents in the IVT labeling kit (Affymetrix Inc.). A hybridization cocktail (200 ㎕) containing 15 ㎍ fragmented cRNA was injected into the Genechip Bovine Gene expression Array (Affymetrix Inc). The array was placed in a 45 hybridization oven at 60 rpm for 16 hours. The array was scanned with a GeneChipScanner 3000 (Affymetrix Inc.).

Table 1. Summary statistics of tissue sample for gene expression analysis

Groups	Animal	EBV	Age (Month)	Marbling score (1-7)	IMF (%)
Low	509	0.37	26	2++	7.11
	537	0.2	27	2++	6.02
	554	0.4	27	3	4.88
	670	0.31	28	3	7.36
	691	0.2	28	3	12.04
High	527	1.02	26	7++	24.35
	547	1.015	27	7++	32.49
	586	0.7	31	7++	16.56
	589	0.69	28	7++	26.24
	632	0.415	28	7++	18.81

Data pre-processing. Data quality control and background correction were carried out using the statistical computing language R. All slides were deemed within normal quality standards. Expression intensities on a log2 scale were obtained from the probe level data using the R *affy* package (Gautier *et al.* 2004) for MAS5.0 (Affymetrix 2002), RMA (Irizarry *et al.* 2003) and GCRMA (Wu *et al.* 2003) methods.

GO and Pathway analysis

Annotation of DE probes was performed using the Database for Annotation. Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp). And an R annotation package derived from the Bos Taurus build 4.0. In subsequent text the term "probe" is replaced by "gene". The DE genes were analyzed in the context of their gene ontology (GO) biological process (Gene Ontology Consortium. 2006). Differentially expressed genes in the *m. longissimus dorsi* of high and low marbled Hanwoo were clustered into pathways using the program Pathway studio (Stratagene, La Jolla, CA; Nikitin *et al.* 2003).

Statistical analysis. Genes differentially expressed between the high- and low marbling groups were detected using a moderated t-test in *limma* (Smyth 2004). For real-time PCR validation analysis of the 21 differentially expressed genes we performed regression analysis of gene expression on intramuscular fat content (IMF %).

III. RESULTS AND DISCUSSION

Differentially expressed genes. A total of 136 differentially expressed genes (DEGs) were detected in 3 data-processing methods; MAS5.0 (65 transcripts), RMA (37 transcripts) and GCRMA (34 transcripts). Of 136 DEGs, 21 were shown to be significant in at least 2 of the summarization methods. Of the 21 differentially expressed genes listed in Table 2, 8 DEGs were identified as up-regulated in the high marbling group and the remaining 13 DEGs were down-regulated in the low marling group.

Table 2. Differentially expressed genes in *M. longissimus dorsi* between high and low marbled Hanwoo

Probe ID	Gene Names	Fold Change	Significance in MAS, RMA and GCRMA
Bt.5323.1.S1_at	SH3 domain YSC-like 1	0.818	* * *
Bt.15675.1.S1_at	ADAM metallopeptidase with thrombospondin type 1	0.953	* * *
Bt.21021.1.S1_at	TBC1 domain family, member 7	0.712	* * *
Bt.2933.1.S1_at	Hypothetical protein LOC788205	0.668	* * *
Bt.9767.1.S1_a_at	Squalene epoxidase	0.867	* * *
Bt.621.1.S1_at	Cytochrome P450, family 51, subfamily A	0.525	* * ns
Bt.23903.1.A1_at	Unknown	-0.53	* * ns
Bt.22362.1.S1_at	Similar to SH3-domain kinase binding protein 1	-0.96	* * ns
Bt.16752.1.A1_at	ATP binding protein	-0.693	ns * *
Bt.1020.1.S1_at	Similar to CDC-like kinase 1	-0.408	ns * *
Bt.19107.2.A1_at	Transcribed locus	-0.548	ns * *
Bt.28011.1.S1_at	Unknown	-1.066	ns * *
Bt.22718.1.A1_at	Proteasome (prosome, macropain) activator subunit 4	-0.326	ns * *
Bt.19107.1.S1_at	Transcribed locus	-0.642	ns * *
Bt.25102.1.S1_a_at	Hypothetical LOC509649	-0.496	ns * *
Bt.22038.1.S1_a_at	Similar to Arginyl-tRNAsynthetase	-0.215	ns * *
Bt.21268.1.S2_at	Ribosomal protein S6 kinase, 70kDa, polypeptide 1	0.459	ns * *
Bt.13342.1.S1_at	Similar to Src-associated protein SAW	-0.383	ns * *
Bt.344.1.S1_at	Major histocompatibility complex, class II, DM alpha	-0.595	ns * *
Bt.21827.2.S1_at	Thimetoligopeptidase 1	-0.818	* * ns
Bt.21794.1.S1_at	Hypothetical protein LOC777601	1.1243	* * ns

GO and Pathway analysis. GO terms were annotated onto the GO database (http://www.geneontology.org). The GO biological process is assigned to 10 categories at level 3 (Figure 1.A). Pathway analysis detected 5 main

pathway "hubs" (*SH3KBP1*, *THOP1*, *ADAMTS4*, *CYP51A* and *SQLE*). Particularly, pathway analysis demonstrated that *ADAMTS4* is activated by immune response related single molecules (*IL-17A*, *TNF*, *NF-kB* and *IL-1* family) and *transforming growth factor beta 1* (*TGFβ1*) (Figure 1B). The pathway results suggest a biological pathway relating to *CYP51A*, *SQLE* and *ADAMTS4* that has not been demonstrated previously in bovine gene expression studies on MAR.

CONCLUSIONS

Microarray analysis identified 21 differentially expressed genes (DEGs) in muscle with divergent marbling phenotype. Pathway analysis for 21 DEGs showed 5 unique pathway hubs associated with steroid biosynthesis, cholesterol metabolism and common transcriptional factors in lipid metabolism. These biological pathways might be represented a phenomenon occurring in muscle with highly divergent marbling phenotype. Of these 5 main pathways, *ADAMTS4* gene was involved in protein catabolic process (GO0006516), which is biologically related to connective tissue degradation that is observed in highly marbled muscle. Pathway analysis revealed that *ADAMTS4* gene is activated by three regulators; *IL-17A*, *TNFα*, and *transforming growth factor β 1 (TGFβ1)* that have not been considered to be associated with marbling fat. We conclude *ADAMTS4* gene might be one of key gene controlling between marbling fat deposition and connective tissue degradation through a complicating biological pathway in skeletal muscle. Further study should be necessary for unveiled biological function of the pathway (*ADAMTS4/TGFβ1*).

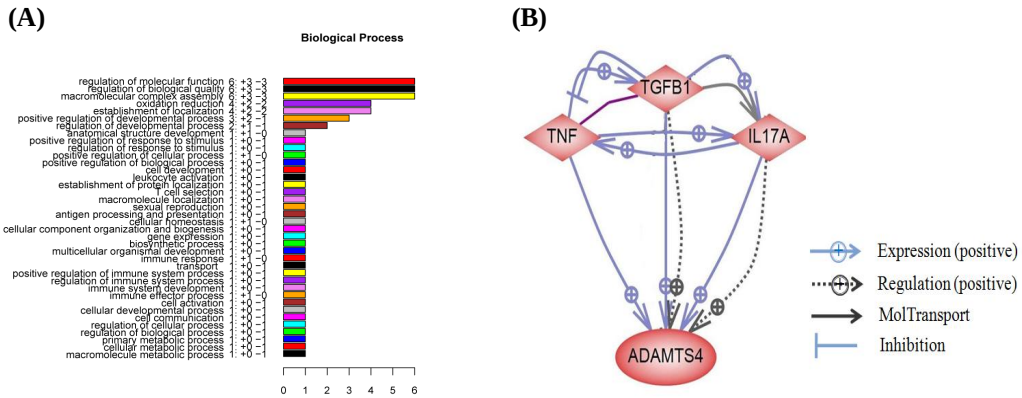

Figure 1. (A) Distribution of the 136 differentially expressed genes on intramuscular fat (IMF) in three data-processing methods (MAS5.0, RMA and GCRMA). (B) GO annotation (biological process term) for 21 differentially expressed genes.

ACKNOWLEDGMENTS

This study was supported by the International Collaborative research fund (Grant No: 200712A01032083) between the Rural Development Administration (RDA) in Korea and Co-operative Research Centre for Beef Genetic Technologies in Australia. Seung Hwan Lee held an International Postgraduate Research Scholarship (IPRS).

REFERENCES

Affymetrix (2002) Statistical algorithms description document

AOAC (1990) Association of Official Analytical Chemists, Washington, DC, USA

Fruhbeck, G., Gomez-Ambrosi, J and Burrell, M.A (2001) Am J Physiol. 280, E827-847

Gautier, L., Cope, L., Bolstad, B.M., Irizarry, R.A (2004) Bioinformatics 20, 307-315

Hocquette, J.F., Ortigues-Marty, I, Pethick, D and Fernandez, X (1998) Livest Prod Sci. 56, 115-143

Kokta, T.A, Dodson, M.V., Gertler, A and Hill, R.A (2004) Domes Anim Endocrinol 27, 303-331

Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Speed, T.P (2003) Nucleic Acids Research 31, e15

Nishimura, T., A. Hattori and K. Takahashi (1999) J Anim Sci, 77, 93-104.

Pethick, D.W., Harper, G.S., Hocquette, J.F and Wang, Y.H (2006) Aust beef-the leader conference, 103-110

Shin, S.M et al (2003) FEBS Lett. 543, 25-30

Smyth, G.K (2004) Statistical Applications in Genetics and Molecular Biology 3(1) 3

Sorisky, A (1999) Crit Rev Clin Lab Sci. 36, 1-34

Wu, Z., et al (2003) Journal of the American Statistical Association 99, 909-917.