Expression of Adiponectin and its Related Genes in *Sancheong Berkshire* Pigs

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***Abstract*—*Sancheong Berkshire* pigs are famous for their excellent marbling (intramuscular fat). We aimed find any relation of Adiponectin, AdipoR1, AdipoR2, and A-FABP with fat accretion in growing pigs though mRNA and protein expression analysis. We also studied the tissue distribution of these genes in *Sancheong Berkshire* pig, and identified that the mRNA expression patterns of adiponectin and A-FABP were similar between the backfat of the growing *Sancheong Berkshire* and that of *Yorkshire pigs*, showing relatively high expression in all growth stages. However, the mRNA expressions of AdipoR1 and AdipoR2 in the backfat of the pigs were increased in an age-related manner that was quite different from those of the *Yorkshire*. Contrary to the previous report, AdipoR1 and R2 were highly expressed in the adipose tissue of *Sancheong Berkshire* pigs. These results suggest that there are breed differences regarding the expression of AdipoR1 and AdipoR2.**

***Index Terms*—Adiponectin; Adiponectin receptors; A-FABP; Fat**

I. INTRODUCTION

The main role of adipose tissue in animals is to store energy. However, it has also been found that adipose tissue is a sort of endocrine organ producing a number of proteins responsible for a variety of biological activities, including leptin, resistin, adiponectin and other factors (Fantuzzi, 2005; Tilg and Moschen, 2006). Some adipocytokines such as adiponectin and leptin account for the association between fat and its related diseases. Additionally, the largest number of such adipocytokines is produced predominantly by adipose tissues. The adiponectin is abundantly expressed in adipose tissue and plays several important roles of inhibiting gluconeogenesis (Combs et al., 2001), facilitating fatty acid oxidation (Fruebis et al., 2001) and enhancing insulin sensitivity in rats (Yamauchi et al., 2002). Circulating adiponectin levels are decreased in individuals with type 2 diabetes (Hotta et al., 2000), coronary artery disease (Lautamaki et al., 2007) and obese human subjects (Arita et al., 1999). Adiponectin receptors 1 (AdipoR1) and 2 (AdipoR2) serve as receptors for globular and full-length adiponectin and mediate increased AMPK, PPARα ligand activities, fatty acid oxidation, and glucose uptake by adiponectin (Kadowaki et al., 2006). Adiopcyte-fatty acid binding proteins (A-FABP) account for approximately 1% of cytosolic proteins in human adipose tissues. (Hotamisligil et al., 1996) suggested that A-FABPs were closely associated with obesity owing to their insulin resistance. The purposes of this study were to investigate existence of any genetic variation of Adiponectin, adipoR1, AdipoR2 and A-FABP genes in the *Sancheong Berkshire* pig, which is famous for its excellent marbling (intramuscular fat), to determine tissue distribution of the four genes, and to establish whether the relative expression of the four genes in tenderloin and backfat varies in growing *Sancheong Berkshire* and *Yorkshire* pigs.

II. MATERIALS AND METHODS

Animal and sample collection

Sancheong berkshire and *Yorkshire* (from Sungchuk Farm, the lineage formed with the pigs from Kagoshima) female pigs whose body weight reached 60, 80, and 110kg were butchered three times, and their tissues were immediately taken, soaked into liquid nitrogen, and kept in a freezer at -80℃ until total RNA isolation.

RNA Extraction and cDNA preparation

Total RNA was isolated from collected samples using Trizol reagent according to the manufacturer's instruction (Life Technologies, Invitrogen). 2ml of Trizol reagent was added to 0.1 ~ 0.2 g of tissue grounded and mixed well with homogenizer, and a 1 ml aliquot of the mixture was transferred into a 1.5ml E-tube. Then, the mixture was left alone at RT for 10 minutes and

centrifuged at 12,000rpm for 10 minutes, and the solution excluding the cell debris was saved into a new tube. 200 µl of chloroform was added to the supernatant, and then 500 µl isopropanol was added to precipitate the RNA. The RNA was washed with 70% ethanol, and dried at RT. The extracted RNA was dissolved in water and quantified spectrophotometrically at 260nm. For each sample, an RNA aliquot was subjected to electrophoresis in 1.5% agarose gel to verify its integrityand.

Cloning of the A-FABP, adiponectin, AdipoR1 and AdipoR2 genes

Adiponectin, adipoR1, AdipoR2 and A-FABP genes were isolated from a fat cDNA library that was constructed using a cDNA Library Kit (Invitrogen, USA). The isolated cDNAs were cloned into a pBluescript vector. Transformation of the cloned cDNAs into *Escherichia coli* XL1-blue cells was done according to the protocol of Mendel and Higa (1970). A Plasmid Mini-prep Kit (QIAGEN, USA) was used for isolation of the recombinant plasmid. The isolated plasmids were digested with *Xho* I and *Eco*RI to view the expected insert on a 1% agarose gel by electrophoresis.

Semi-quantitative RT PCR

For RT-PCR, the first strand cDNA was synthesized by using Superscript II Reverse Transcriptase, according to the manufacture's protocol (Invitrogen, USA). Briefly, a 5 of extracted RNA was added to a reaction mixture consisting of 4 ㎕ of 5X First strand Buffer (Invitrogen, Carlsbad, CA), 1 ㎕ of 10mM dNTPs (Promega, USA) dissolvedDEPC-water, 2 ㎕ 0.1M DTT (Invitrogen, USA), 1 ㎕ (200U/㎕) of SuperScript ReverseTranscriptase II (Invitrogen, USA), 1 ㎕ (0.5 ㎍/㎕) of oligo-d(T) 12-18 primer (Invitrogen,USA), 1 ㎕ of RNase Inhibitor (Invitrogen, USA), and RNase-free water. Then, the RT step was carried out at 42℃ for 1 hr, followed by heating at 70℃ for 15 min and adding 1 ㎕ RNase H at 37℃ for 20 min before storage at 4℃. A set of negative control was also included except for the reverse transcription reaction. For internal control for assessing the relative amount of target gene from different tissue samples, the GAPDH gene was used for the comparison (Table 1). RT-PCR products of the four genes were separated on a 2% TAE agarose gel and visualized by UV after ethidium bromide staining.

Western immunoblotting

Isolated tissues were homogenized in ice cold 0.25M sucrose containing 1mM dithiothreitol and 1mM EDTA. The mixtures were centrifuged at 100,000 x g for 1hr at 4˚C, and the cytosolic fractions were collected and stored at -80˚C. Protein content of the cytosolic fractions was determined using the bicinchoninic acid assay with BSA as standard. The cytosolic proteins were separated by electrophoresis on 15% SDS–PAGE gels and were then transferred to PVDF membranes. The membranes were saturated with 5% non-fat dry milk in TBS/Tween overnight at 4℃ and, after washing with TBS/Tween, were probed with adiponectin, adipoR1, adipoR2 pAb (1:3000 dilution in TBS/Tween with 5% BSA) for 3 h at room temperature. Then the blots were washed three times with TBS/Tween and incubated with a goat anti-mouse secondary antibody conjugated to HRP (1:3000 dilutions in TBS/Tween). The washed blots were treated with ECL reagents according to the manufacturer's instructions, and the bands were visualized luminographically on X-ray films (Kodak).

III. RESULTS AND DISCUSSION

Cloning of the adiponectin, A-FABP, adipoR1 and adipoR2 genes from Sancheong Berkshire pig A-FABP, adiponectin, adipoR1 and R2 genes were cloned from the fat cDNA library of the Sancheong Berkshire pig to find any genetic variation compared with the porcine sequences deposited in GenBank. The cDNA sequences for the respective genes were as follows: 920bp for adiponectin; 230bp for A-FABP; 1144bp for AdipoR1; and 1015bp for AdiopR2. The genes were sequenced with the gene specific primers (Table.1). The sequences of the four genes were identical to those registered in GenBank:adiponectin (AY589691); A-FABP (AF102872); AdipoR1 (AY578142); AdiopR(NM001007192). Expression of adiponectin, A-FABP and adiponectin Receptor 1 and 2 in muscle and adipose tissues of growing pigs The expression of four genes were assessed by RT-PCR analysis and Western immunoblotting in the muscle (tenderloin) and adipose tissue (backfat) from Sancheong Berkshire and Yorkshire pigs by growth stages (60kg, 80kg and 110kg in weight). The mRNA expression level of adiponectin and A-FABP had higher in the adipose tissue than muscle, and had highest expression level in the adipose tissue at the late growth stages (weighing 110kg) (Figure 1). We performed statistical analysis on these results using SAS program. The expression levels of two genes had no statistical significances in the growth stages and the strains. However, the mRNA level of each gene

was significantly over-expressed in adipose tissue than muscle ($P<0.01$) (Figure 2–(A), (B)). The protein level of adiponectin was higher in the tenderloin and adipose tissue, and was not correlated with the growth stage nor the strains as mRNA expression level (Figure 5 – (A)). The mRNA expression level of AdipoR1 was similar to that of AdipoR2. Both of genes were highly expressed in adipose tissue at 60kg and 110kg of Yorkshire pigs, and at 110kg of Sancheong Berkshire. The expression levels of AdipoR1, AdipoR2 were decreased with growth in the muscle, however increased with growth in the adipose tissue. A statistical analysis on RT-PCR results was carried out to identify the correlation within various parameters (species / growth stage / tissue) through testing the statistical significance of interrelationship between the expression levels of genes and two or more variables (Figure 2 – (C), (D)). We found that the expression levels of AipoR1, AdipoR2 were statistically significant in all varieties ($p<0.01$ and $p<0.05$ respectively). The protein expression level of AdipoR1 and AdipoR2 were similar to the expression level of mRNA (Figure 5 – (C), (D)). Tissue distribution of Adiponectin, A-FABP and AdipoR1 and AdipoR2 The mRNA expression of adiponectin, A-FABP and AdipoR1 and AdipoR2 was assessed by RT-PCR analysis and Western blotting in various tissues from Sancheong Berkshire pigs weighing 110kg. (Figure3, Figure5). The mRNA expression level of adiponectin and A-FABP were highest in adipose tissue (Figure 4 – (A), (B)). Adiponectin protein was detected at highest level in adipose tissue and was also detected relatively high level in muscle (Figure 5 – (B)). The expression levels of AdipoR1 and AdipoR2 were highest in adipose tissue. AdipoR1 mRNA was expressed higher in heart than liver, however the expression of AdipoR2 was higher in liver than heart (Figure 3, Figure 4 – (C), (D)). Western blot analysis showed that these two receptors were detected at higher level in adipose tissue than muscle (Figure 5 – (D), (F)). Correlation analysis among genes To analyze any relationship among adiponectin, A-FABP, AdipoR1, and AdipoR2, we performed SAS between the gene expression and tissues. We found that there was a linear relationship among the expression of genes in the muscle and adipose tissue (Table 2). In the muscle tissue, adiponectin was correlated with A-FABP ($r=0.72351$) and highly correlated with AdipoR1 ($r=0.83062$). A-FABP was correlated with AdipoR1 ($r=0.71367$). AdipoR1 and AdipoR2 showed relatively high correlation ($r=0.81491$). In the adipose tissue, adiponectin was correlated with A-FABP ($r=0.67371$). AdipoR1 and AdipoR2 were highly correlated to each other ($r=0.96501$).

IV. CONCLUSION

We attempted to perform expression profiling with the four genes isolated from Sancheong Berkshire pigs and to clarify an association between their expression profiles and the quality of meat. Our results clearly showed that there were age250 related differences of four genes expression levels in intra-muscle tissues between two breeds. AdipoR1 and AdipoR2 were dominantly expressed in adipose tissues of Sancheong Berkshire pig. Whether these expression patterns are specific in Sancheong Berkshire pig and which gene(s) might contribute the quality of meat await further investigation.

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