HEAT SHOCK PROTEIN BETA 1 WAS ASSOCIATED WITH INTRAMUSCULAR FAT CONTENT IN LONGISSIMUS MUSCLE OF KOREAN CATTLE

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***Abstract*—In previous proteomic studies, heat shock protein beta 1 (HSPB1), succinate dehydrogenase (SDH) and triosphosphate isomerase (TPI) were discovered as candidate proteins related with meat qualities in cattle. For the association study with expression levels and intramuscular fat content in m. *longissimus* tissue of Korean cattle (Hanwoo), ten steers of each group with low-marbled group (7.44±0.463%) and high-marbled group (23.54±0.881%) were examinedby immunoblotting, real-time PCR and statistical analyses. In present study, HSPB1 expression in both mRNA and protein level was significantly down-regulated according to intramuscular fat content increased (P<0.001). Otherwise, protein expression of SDH was more highly expressed in high-marbled samples (P<0.01). The HSPB1 and SDH were also shown to have significant effect on intramuscular fat content (P<0.05). These results suggest that HSPB1 may be good candidate as marker for identifying fat content in *longissimus* muscle of Hanwoo steer.**

***Index Terms*—Intramuscular fat, Korean cattle, HSPB1**

I. INTRODUCTION

In the Korean beef industry, the tenderness, flavor, and marbling of meat are very important factors that determine their economic value. Among these factors, marbling is particularly coveted by Korean consumers and producers. Therefore, it is important to identify the genes that regulate intramuscular fat so that marker-assisted selection (MAS) can be used to target these characteristics in meat production. Meat qualities, in particular, marbling are controlled by multiple gene expression. To date, transcriptomic and proteomic studies for phenotypic profiling involving the use of microarray and two-dimensional electrophoresis have identified genes and proteins associated marbling and tenderness in cattle (Mullen, Stapleton, Corcoran, Hamill & White, 2006). Other studies more specific, selected candidates according to these molecular functions were used in studies for providing a better understanding of muscle physiological processes and their influence on meat quality. These studies demonstrated that analysis of expression was very important for understanding of molecular mechanisms associated to phenotypic characters in animals.

Previously, we have identified three candidate proteins such as heat shock protein beta 1 (HSPB1), succinate dehydrogenase (SDH) and triosphosphate isomerase (TPI) in m. *longissimus* muscle in low and high-meat quality groups or fattening stages of Hanwoo steers (which the steers have different intramuscular fat contents) using proteomic study (Kim et al., 2008; Kim et al., 2009). However, relationship between expression value and intramuscular fat content was not clear. Therefore, to determine whether the candidates were associated with intramuscular fat content, immunoblotting, real-time PCR and statistical analyses were performed in *longissimus* muscle with low-(n=10) and high-marbled (n=10) samples.

II. MATERIALS AND METHODS

Animals and sample preparation

Twenty samples of m. *longissimus dorsi* muscle from low- and high-marbled Hanwoo (n = 10 animals of each group) steers were obtained at the junction between the 11th and 12th lumbar vertebrae within 30 min after slaughter. The selected tissues were placed in liquid nitrogen, ground to a fine powder using a mortar, and stored at -80°C. All experimental procedures and care of animals were conducted in accordance with the guidelines of the Animal Care and Use Committee of the National Institute of Animal Science in Korea.

Measurement of meat qualities

The fat content in m. *longissimus* muscle was analyzed using the methods of the Association of Official Analytical Chemists (AOAC) (Feldsine, Abeyta & Andrews, 2002). Objective meat color (CIE L, a, b) was determined using a Minolta Chromameter CR300 (Minolta, Japan) on freshly cut surfaces of meat samples after a 30 min blooming at 1°C.

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Warner-Bratzler (WB) shear force (kg) was measured on cooked steaks (2.54 cm thick) in a pre-heated water bath for 60 min or until the core temperature reached 70℃ and subsequently cooled in running water (ca. 18℃) for 30 min to reach a core temperature below 30℃. Eight cores of 1.27 cm diameter were made for each sample, and peak force was determined using a V-shaped shear blade with a cross-head speed of 400 mm/min (Wheeler, Shackelford & Koohmaraie, 2000).

Table 1. Characteristics of meat quality traits between low- and high-marbled groups (Mean ± S.E)

Group	Age (months)	CIE			CW (kg)	IMF (%)	WBS (kg)
		L	a	b			
Low	27.1 ± 0.276	35.11 ± 0.548	21.18 ± 1.079	9.66 ± 0.701	409.2 ± 10.588	7.44 ± 0.463	5.07 ± 0.332
High	29.5 ± 0.477	37.54 ± 0.635**	21.50 ± 0.355	9.62 ± 0.225	478.17 ± 13.618	23.54 ± 0.881***	3.49 ± 0.307*

CW, carcass weight; IMF, intramuscular fat content; WBS, Warner-Bratzler shear force. *, **, *** Significant differences (P < 0.05, 0.01 and 0.001) between low- and high-marbled groups determined using the mixed ANOVA module.

Quantitative real-time PCR

The mRNA levels were analyzed by real-time PCR using gene-specific primer set. Each gene primers were designed using PrimerQuest (http://eu.idtdna.com/Scitools/Applications/Primerquest/). Total RNA was prepared from each tissue sample (100 mg) with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and then purified using RNeasy MinElute Clean-up kit (Qiazen, Valencia, CA, USA). RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo scientific, USA). The RNA purity (A_{260}/A_{280}) was over 1.90. For cDNA synthesis, 2 µg RNA was reverse transcribed in a 20 µl reaction volume using random primers (Promega, Madison, WI, USA) and reverse transcriptase (SuperScript II Reverse Transcriptase, Invitrogen Life Technologies). Reactions were incubated at 65℃ for 5 min, 42℃ for 50 min, and then 70℃ for 15 min to inactivate the reverse transcriptase.

Real-time PCR was performed using the 2X Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) with the 7500 Real Time PCR system (Applied Biosystems) using 10 pM of each primer. The PCR was run for 2 min at 50℃ and 10 min at 95℃, followed by 40 cycles of 95℃ for 10 s, and then 60℃ for 1 min. Following amplification, a melting curve analysis was performed to verify the specificity of the reactions. The end point used in the real-time PCR quantification, Ct, was defined as the PCR threshold cycle number.

For selection and using of internal control genes, we calculated gene-stability (M) value and finding the two genes among the commonly used housekeeping (HK) genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (ß-actin), ribosomal protein, large, P0 (RPLP0) and 18S ribosomal RNA (18S rRNA) using the *geNorm* analysis. The RPLP0 gene showed the lowest M value and best combination of two genes were RPLP0 and ß-actin. The M value for best combination of two genes was 0.393, which met the stability requirement to be an HK gene. Therefore, RPLP0 and ß-actin genes were selected as the internal control for normalization (Vandesompele et al., 2002).

Immunoblotting

For protein extraction from individual samples, frozen muscle tissue (100 mg) was incubated for 40 min in 1 ml of 8 M urea, 2 M thio-urea, 65 mM dithiothreitol (DTT) and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Samples were centrifuged at 40,000 × *g* for 30 min and the supernatants were used as the protein extract. Protein concentration was determined using the protein assay system (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard. For western blotting, 30 µg of sample proteins were separated on SDS-PAGE according to the method of Laemmli (Laemmli, 1970) and gels were transferred to PVDF membranes (Millipore, MA, USA) in ice-cold transfer buffer (25 mM Tris-Cl, pH 8.3, 1.4% glycine, 20% methanol) at 250 mA for 60 min. Membranes were treated with blocking buffer containing 3% non-fat milk (Becton, Dickinson and Company, MD, USA) in TBS/T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and incubated overnight at 4℃. Primary goat anti-HSP27 (sc-1048), SDH (sc-59687) and TPI (sc-22031) antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), were used at 1:200 dilutions in TBS/T with 3% non-fat milk. Following 2 h of incubation with primary antibodies, membranes were washed three times for 10 min each with 10 ml of TBS/T. Horseradish peroxidase-labeled (HRP) anti-goat secondary antibody was diluted 1:1,000 in TBS/T with 3% non-fat milk and incubated for 2 h. After three 10 min washes, membranes were visualized using a chemiluminescent HRP substrate (Millipore) and a VersaDoc image analyzer (Bio-Rad). The band densities were calculated by Quantity One software (Bio-Rad, Ver. 3.1) and normalized by density of α-tubulin (sc-12462, Santa Cruz Biotechnology, Inc.).

Statistical analysis

A linear regression model was used to examine the association between intramuscular fat contents and expression of protein and gene level using the R Statistical Package (Team, 2006). This resulted in the following equation:

$Expression_{ij} = \mu + IMF_i + Age_j + e_{ij}$

where Expression is an expression value of gene and protein, µ is overall mean, IMF is intramuscular fat content (%) of each animal and Age is slaughtering age (months) as a covariate. We also examined their least square means (LSM) for testing if expression level was significant difference between low- and high-marbled group using Student`s t-test.

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III. RESULTS AND DISCUSSION

In present study, we investigated expression levels of three candidates, heat shock protein beta 1 (HSPB1), succinate dehydrogenase (SDH) and triosphosphate isomerase (TPI) in m. *longissimus* muscle between low- and high-marbled groups of Hanwoo (Korean cattle) steers. Ten steers with the low intramuscular fat content (low-marbled group) and ten steers with the high intramuscular fat content (high-marbled group) were used in this study for association with expression in mRNA and protein levels and intramuscular fat content. In meat qualities, such as lightness (L*) value, carcass weight, intramuscular fat content and Warner-Bratzler shear force, were different between two groups. In particular, the average intramuscular fat content in *longissimus* muscle for low-marbled group was 7.44±0.463% and for high-marbled group was 23.54±0.881% (Table 1). To confirm the differences in low- and high-marbled groups, the ANOVA analysis was used by the mixed procedure of R statistical package for animal nested within age as the random effect. As shown in Table 1, lightness (L*) value ($P < 0.01$), intramuscular fat content ($P < 0.001$) and Warner-Bratzler shear force ($P < 0.05$) were significantly difference between the groups. There were no significant differences between the low- and high-marbled groups for the properties of redness (a*), yellowness (b*) value and carcass weight traits (Table 1).

Table 2 displays the differences of individual expression of both gene and protein levels between the groups. Interestingly, HSPB1 ($P < 0.01$ and 0.001, respectively) were significantly different at the level of gene and protein between low- and high-marbled groups. The mRNA and protein level of HSPB1 in high-marbled group was approximately 2.0 and 2.2 times lower than that in the low-marbled group (Figure 1). The SDH protein also was significantly different ($P < 0.001$, respectively) between the groups. In the protein level, SDH was higher expressed in the high-marbled group (approximately 2.0 times), while mRNA level was not different between the groups. We examined their least square means (LSM) for testing if there was significant difference between low- and high-marbled groups using Student`s t-test. These results also showed that HSPB1 had a low expression pattern in the high-marbled group (Table 3).

Table 2. ANOVA table for each gene and protein associated with intramuscular fat (IMF) content in Hanwoo steers

Real-time PCR							Immunoblotting						
Gene	Source	Df	Sum. Sq.	Mean Sq.	F value	Pr(>F)	Protein	Source	Df	Sum. Sq.	Mean Sq.	F value	Pr(>F)
HSPB1	IMF	1	0.05709	0.05709	8.529	0.00954**	HSPB1	IMF	1	695.86	695.86	22.823	0.000175***
	Age	1	0.000113	0.000113	0.0168	0.89831		Age	1	41.95	41.95	1.376	0.25696
	Residuals	17	0.113792	0.006694				Residuals	17	518.32	30.49		
SDH	IMF	1	0.00017	0.00017	0.0547	0.8178	SDH	IMF	1	2753.13	2753.13	19.567	0.000372***
	Age	1	0.000023	0.000023	0.0075	0.9318		Age	1	147.96	147.96	1.052	0.13952
	Residuals	17	0.052659	0.003098				Residuals	17	2391.94	140.7		
TPI	IMF	1	0.000093	0.000093	0.0324	0.8594	TPI	IMF	1	44.637	44.637	4.157	0.05733
	Age	1	0.000027	0.000027	0.0094	0.924		Age	1	1.217	1.217	0.113	0.7405
	Residuals	17	0.049013	0.002883				Residuals	17	182.551	10.738		

Table 3. Least squares mean (± standard error) and t-statistics for expression levels between low- and high-marbled groups in Hanwoo steers

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Real-time PCR				
Name	Low	High	t value	Pr>[t]
HSPB1	0.23 ± 0.021	0.10 ± 0.021	3.224	0.0047**
SDH	0.12 ± 0.015	0.10 ± 0.015	0.538	0.5970
TPI	0.23 ± 0.014	0.22 ± 0.014	0.185	0.8550
Immunoblotting				
Name	Low	High	t value	Pr>[t]
HSPB1	19.53 ± 2.283	11.06 ± 2.283	4.303	0.0004***
SDH	16.37 ± 4.992	43.92 ± 4.992	-3.793	0.0013**
TPI	5.69 ± 1.361	3.79 ± 1.361	1.374	0.1861

Heat shock proteins (HSPs) have essential roles in the synthesis, transport and folding of proteins (Schlesinger, 1990) and are often referred to as molecular chaperones. Previously reports demonstrated that heat shock proteins, especially DNAJA1 and HSPB1 were correlated with beef tenderness, which the proteins showed a negatively related to WB-shear force (Bernard et al 2007, Morzel, Terlouw, Chambon, Micol & Picard, 2008). Other report, HSP27 interacts with the insulin-like growth factor receptor (IGFR) 1 and its signal transducer, the serine/threonine kinase protein Akt, thereby influencing insulin sensitivity (Rane et al., 2003). The IGFR-1 also triggers intracellular cascades which induce

or repress transcription factors that modulate adipogenesis. These results demonstrated that HSPB1 may be indirectly modulate adipogenesis by interacts with IGFR-1 and its signaling.

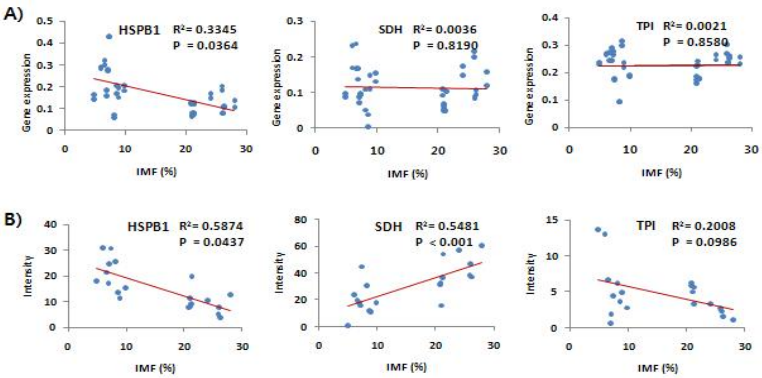

Figure 1. Regression analysis between expression level from real-time PCR (A) Western blotting (B) and intramuscular fat content (%) for each sample. HSPB1, heat shock protein beta 1; SDH, succinate dehydrogenase; TPI, triosephosphate isomerase.

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IV. CONCLUSION

We confirmed that the expression levels of heat shock protein beta 1 (HSPB1) by both real-time PCR and immunoblotting were correlated well with intramuscular fat content. Our results suggest that HSPB1 may be useful as biomarker for intramuscular fat content in Korean cattle (Hanwoo) steer.

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