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Identification of marbling-related candidate genes in *M. longissimus dorsi* of high- and low marbled Hanwoo *(Korean Native Cattle)* steers

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This study was conducted to identify marbling-related candidate genes in *M. longissimus dorsi* of high- and low-marbled Hanwoo. The *longissimus dorsi* muscles were selected for gene expression from eight Hanwoo steer carcasses based on crude fat content. In the analysis of variance, gene expression of five candidate genes, FABP4, SCD, PPAR γ , Titin and Nebulin was determined to be significantly different between high- and low-marbled Hanwoo steers ($P < 0.0001$). The Pik-4 and CaMK II genes were also shown to have a significant effect on crude fat content ($P < 0.01$). In the analysis of the differential expression between high- and low marbled groups, FABP4 gene expression was approximately 2 times higher in the high marbled group relative to the low marbled group. However, the PPAR γ and SCD gene were highly expressed in the low marbled group. In addition, Titin and Nebulin were highly expressed in the low marbled group when placed under relatively high shear force. Finally, the Pik-4 and CaM K II gene also displayed a high expression pattern in the low marbled group. [BMB reports 2008; 41(12): 846-851]

INTRODUCTION

Marbling (intramuscular fat) is a main factor in determining important parameters that contribute to meat quality, such as the juiciness and flavor of beef. It is also an essential contributor to the economic value for producer in the Korean beef market. To improve the marbling of an animal, a grain based feeding system is performed in the Korean finishing farm sector. However, marbling can still increase as a result of other fat depot, such as subcutaneous fat and visceral fat, which leads to inefficient meat production. Therefore, genomic studies have been performed to identify marbling-specific genes for application in animal breeding programs. Marbling is known as a late maturing trait. However, fat development in muscle begins at an early stage of animal development and intramuscular fat content at these early stages are an essential factor in determining the final level of marbling in the adult animal (1). In Hanwoo cattle, intramuscular fat content significantly increased between 12 and 30 month of age (2). Over this 18 month period, the muscle crude fat content increased from 7% to approximately16% (2). During this period, many metabolic events involving fatty acid trafficking, oxidation of fatty acid in mitochondria or storage takes place in muscle tissue to balance TAG synthesis (3). Therefore, the differences in metabolic events that occur within the muscle tissue may be a good indicator of marbling production (3).

Based on this notion, we have previously identified 11 differentially expressed genes in *longissimus dorsi* in the early (12 month) and late (27 month) stages of Hanwoo steers development (during which the steers have undergone dramatic intramuscular fat accumulation) using a differential display RT-PCR analysis (4, 5).

Therefore, the objective of this study was to identify marbling-related genes in *M. longissimus dorsi* of high- and low marbled Hanwoo steers. To achieve this objective, eight *M. longissimus dorsi* were selected based on the crude fat contents of the muscle tissue (high group (n = 4) is 16-32%; low (n = 4) is 4-7%) and the estimated breeding value (EBV) of the marbling score. We then carried out gene expression analysis of 11 previously identified differentially expressed genes in *longissimus dorsi* of high- and low marbled Hanwoo steers using real-time PCR. We examined the effect of the marbling group on individual gene expression using an analysis of variance (ANOVA) model.

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Table 1. Summary of animal and carcass data for gene expression analysis

Groups	ID	Factors			Traits	
		Treatment*	Age (month)	Crude fat# (%)	Shear force# (Kg)	Marbling score
Low	509	T3	26	7.11	7.57	2++
	537	T2	27	6.02	4.90	2++
	554	T3	27	4.88	5.50	3
	670	T2	28	7.36	4.69	3
High	527	T2	26	24.35	2.74	7++
	547	T3	27	32.49	2.84	7++
	586	T2	31	16.56	2.89	7++
	589	T3	30	26.24	2.90	7++

#Correlation between crude fat contents and Shear force is -0.78
*Treatment: T1; TDN standard (70% (6-12 month)-70% (12-18 month)-71% (18-24 month)-72% (24-26 month); T2; TDN standard (70% (6-12 month)-71% (12-18 month)-72% (18-24 month)-73% (24-26 month); T3; TDN standard (70% (6-12 month)-72% (12-18 month)-73% (18-24 month)-74% (24-26 month)

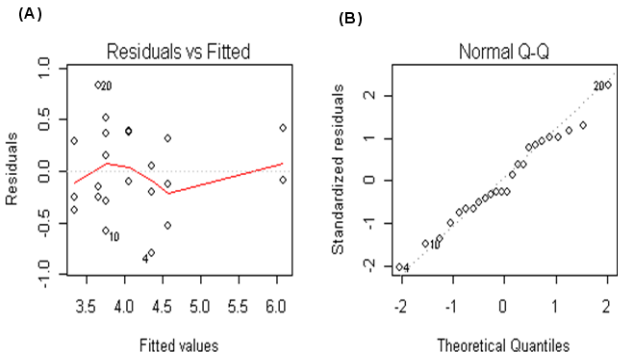

Fig. 1. Diagnostics of linear models for the fatty acid binding protein 4 gene. (A) and (B) are diagnostic residual plot and Q-Q plot of this linear model.

RESULTS AND DISCUSSION

Table 1 summarized the variation factors and traits for 8 selected Hanwoo. The difference in crude fat content between high and low marbled Hanwoo steers was 18%. In addition, the shear force between the two groups was negatively correlated with the crude fat content of the muscle tissue.

We examined gene expression of 11 genes, which were differentially expressed between 12 and 27 month old in *M. longissimus dorsi* with different crude fat content (4, 5). To verify the validity of this statistic analysis, residuals were analyzed (Fig. 1). Fig. 1 (A) shows that the distribution may not violate the assumption of constant variance. However, the residual plot does imply that the residuals are normally distributed. Of the 11 identified genes, five genes (C/EBP α, PPAR γ, ACL, SCD and FABP4) are known to be related to adipocyte differentiation and fatty acid synthesis. In addition, Titin and Nebulin are a main component of myofibril in muscle. The gene expression intensity of these 11 genes was estimated using the ΔCt method. The ΔCt value was calculated by subtracting the18S rRNA Ct value from the Ct values of individual target genes. The intensity then was transformed using the formula: $2^{-\Delta Ct}$ (Applied Biosystems, USA). The effect of the 11 candidate genes on the marbling score was estimated using an ANOVA model.

Table 2 displays the effect of individual gene expression on the marbling group. The gene expression of the five candidate genes, FABP4, SCD, PPARγ, Titin and Nebulin was significantly different between high- and low-marbling score ($P < 0.0001$). Marbling score also had an effect on Pik-4 and CaMK II gene expression ($P < 0.01$).

To determine if the differences between the high- and low marbled groups were significant, we estimated the least square mean (LSM) of the two groups and performed Student's t-test (Table 3). Unexpectedly, the PPARγ and SCD gene had a high gene expression pattern in the low marbled group. However, there have been some reports that support this result. The PPAR γ gene is known as an early transcriptional factor for adipogenesis and it plays an essential role in triggering fatty acid synthesis related gene expression. Expression of PPARγ and C/EBPα decrease substantially with age and older animals do not support fat synthesis, even though the animal may have a good genetic performance for fat synthesis (6). The SCD gene is associated with fatty acid composition and converts stearic acid into oleic acid. Recently, Taniguch *et al* (7) reported that a non-synonymous SNP in Exon IV of the SCD gene lead to a change in the monounsaturated fatty acid composition in Japanese black cattle. In addition, the SCD enzyme activity was positively correlated with unsaturated fatty acid composition (8). Thus, the SCD gene may be related to fatty acid composition (fat quality) rather than the amount of fat. Therefore, gene expression of PPARγ and SCD might not reflect a positive relationship between gene expression and marbling score.

However, the FABP4 gene had a higher gene expression pattern in the high marbled group relative to the low marbled

Table 2. ANOVA table for each gene associated with crude fat content in Hanwoo steers

Gene	Source	Df	Sum Sq	Mean Sq	F-value	Pr (>F)[1]
ACL	*TR*[2]	1	7.641	7.641	4.423	0.048*
	FAT[3]	1	0.684	0.684	0.396	0.536
	Age	1	29.768	29.768	17.23	0.0004***
	Residuals	20	34.545	1.727		
C/EBPα	*TR*[2]	1	2.686	2.686	1.075	0.312
	FAT[3]	1	6.224	6.224	2.491	0.130
	Age	1	20.534	20.534	8.218	0.009**
	Residuals	20	49.971	2.499		
FABP4	*TR*[2]	1	0.603	0.603	3.44	0.07
	FAT[3]	1	1.705	1.705	9.74	0.005**
	Age	1	13.19	13.19	75.39	0.0001***
	Residuals	20	8.592	0.43		
PPARγ	*TR*[2]	1	0.6474	0.6474	0.6507	0.429
	FAT[3]	1	18.0518	18.0518	18.142	0.0003***
	Age	1	14.9057	14.9057	14.980	0.0009***
	Residuals	20	19.8998	0.995		
SCD	*TR*[2]	1	2.3518	2.3518	11.236	0.003**
	FAT[3]	1	4.5087	4.5087	21.541	0.0001***
	Age	1	26.9033	26.9033	128.53	0.0001***
	Residuals	20	4.1862	0.2093		
PiK-4	*TR*[2]	1	0.007	0.007	0.022	0.883
	FAT[3]	1	2.483	2.483	7.428	0.013*
	Age	1	95.282	95.282	285.09	0.0001***
	Residuals	20	6.684	0.334		
WDNM1	*TR*[2]	1	1.949	1.949	1.163	0.293
	FAT[3]	1	0.131	0.131	0.078	0.782
	Age	1	47.382	47.382	28.28	0.0001***
	Residuals	20	33.503	1.675		
CaM K II	*TR*[2]	1	3.72	3.72	6.619	0.018*
	FAT[3]	1	2.727	2.727	4.851	0.039*
	Age	1	32.534	32.534	57.88	0.0001***
	Residuals	20	11.241	0.562		
Feritin	*TR*[2]	1	5.072	5.072	2.219	0.151
	FAT[3]	1	1.757	1.757	0.768	0.391
	Age	1	79.011	79.011	34.57	0.0001***
	Residuals	20	45.707	2.285		
Nebulin	*TR*[2]	1	46.509	46.509	25.33	0.0001***
	FAT[3]	1	96.227	96.227	52.41	0.0001***
	Age	1	40.192	40.192	21.89	0.0001***
	Residuals	20	36.721	1.836		
Titin	*TR*[2]	1	86.212	86.212	10.06	0.004**
	FAT[3]	1	76.548	76.548	8.934	0.007**
	Age	1	147.15	147.15	17.17	0.0001***
	Residuals	20	171.353	8.568		

[1]Signif.codes: 0.0001'***', 0.001'**', 0.01'*'
[2]TR; Treatment, [3]FAT; crude fat%

group. FABP4 gene plays a role in lipid hydrolysis and fatty acid trafficking in different tissue (9, 10). Hocquete *et al* (3) reported that fatty acid binding protein 4 activity was strongly correlated with intramuscular fat content in a study where the different genetic performance of intramuscular fat deposition were compared between 2 muscle type of three breed with. In addition, Yong *et al*, (11) reported that the FABP4 gene was expressed 2 times higher in longissimus dorsi of the highly marbled Japanese Black (Wagyu) breed than in the Holstein breed. In this study, the FABP4 gene exhibited a similar positive correlation between gene expression and muscle crude fat content as reported by Hocquete *et al* (3).

Recently, a SNP (*7516G>C*:AAFC1136716) in the bovine FABP4 gene has been reported to have a significant effect on the marbling score in Wagyu × Limousin F2 crosses (12). Park *et al* (13) also reported that a non-synonymous SNP ($3678G>A^{Met>Val}$) was associated with the marbling score in Hanwoo (Korean Native Cattle). These results suggest that the FABP4 gene might be a causal gene, which may explain the marbling phenotype variation observed in Hanwoo.

The Titin and Nebulin genes are strongly associated with myofiber and muscle development. Nebulin is a large protein

Table 3. Least squares mean and *t*-statistics for individual gene expression between high-and low marbled group

Gene Name	Least square mean		*t*-values*	*P*-values
	Marbling high (n = 4)	Marbling low (n = 4)		
FABP4	5.58 ± 0.19	3.32 ± 0.19	16.05	0.001
PPARγ	-1.39 ± 0.30	0.99 ± 0.30	-11.18	0.001
SCD	-5.51 ± 0.13	-3.81 ± 0.13	-17.33	0.001
PiK-4	3.23 ± 0.17	4.09 ± 0.17	-6.90	0.001
CaM K II	0.25 ± 0.22	1.84 ± 0.22	-9.92	0.001
Nebulin	-1.11 ± 0.41	4.01 ± 0.41	-17.65	0.001
Titin	-2.68 ± 0.88	2.90 ± 0.88	-8.88	0.001

*t-statistics: Mean diff / SE diff; Mean diff (M) = M_{high} - M_{low}

; SE diff = $\sqrt{\frac{V_{high}}{N_{high}} + \frac{V_{low}}{N_{low}}}$

of the cytoskeletal matrix that consists of the thick and thin filaments within the muscle sarcomeres (14). Titin is also one of the sarcomeric proteins and is involved in the reorganization of the cytoskeleton during myofibrillogenesis (15). In this analysis, Titin and Nebulin were highly expressed in the low marbled group when placed under relatively high shear force (Table 1 and Table 3). These results indicate that Titin and Nebulin are related to meat toughness. In a study by Anderson (16), the loss of several myofibril proteins caused the adipose mass in muscle to increase. In addition, Light *et al* (17) reported that the Titin gene is involved in determining meat toughness.

Finally, we found that the Pik-4 and CaM K II genes also displayed higher expression in the low marbled group. In a previous study, CaMK II gene expression was also determined to be lower in the late fattening stage, which dramatically increases intramuscular fat in cattle (5). To date, there have been no reports where the effects of Pik-4 and CaM KII gene expression on intramuscular fat content in livestock were examined. However, according to the CaM KII gene expression result in this study, it is plausible that the CaM KII gene may be negatively correlated with intramuscular fat contents. Schrauwen (18) suggested that the intramuscular triglyceride content remains low when mitochondria biogenesis related to oxidative capacity is high in muscle. This type of mitochondria biogenesis is accomplished by peroxisome proliferators-activated receptor γ coactivator 1 (PGC-1), a key regulator of *in vivo* mitochondria biogenesis (19). The calcium/calmodulin-dependent protein kinase (CaMK IV) stimulates mitochondria biogenesis via a key regulator of *in vivo* mitochondria biogenesis, PGC-1 (19). Therefore, the calcium/calmodulin-dependent protein kinase II gene may also be involved in this mechanism as one of the regulators of mitochondria biogenesis.

We therefore conclude that FABP4, Titin, Nebulin and CaMK II gene are strongly associated with the marbling score in Hanwoo steers.

MATERIALS AND METHODS

Muscle samples

M. longissimus dorsi samples were taken at the junction between the 11th and 12th lumber vertebrae of cold carcasses (20) from a research project of energy concentrations in concentrates and slaughter age on performance and carcass characteristics in Animal Nutrition and Physiology Division of National Institute of Animal Science (NIAS; 21). All steers were separated into eighteen pens, based on birth weight (BW), and randomly subjected to three TDN (Total Digestible Nutrients) levels of concentrate. For gene expression analysis, eight *M. longissimus* dorsi were selected based on the crude fat% and marbling score EBV values. All TDN levels are listed in Table 1. Five steers at the selected time of TDN treatment were humanely slaughtered (ranging from 26-31 months old) at the slaughterhouse in NIAS, and carcass measurements and chemical fat percentages for all carcass muscle cut were determined. The two levels of TDN treatments and the age of the slaughtered animals selected in this study are shown in Table 1.

Analysis of chemical composition and shear force

Crude fat content in the muscle tissue was measured according to the method described by AOAC (22). Eight muscle samples (2-cm diameter) were taken from the muscle slice after cooking (at 90^{o}C for 1 h). The samples were sheared once through the center using an Instron 3343 (US/MX50, A&D Co., MA, USA) equipped with a Warner Bratzler shearing device. The shear force value (in kilograms) was the mean of the maximum forces required to shear each test of the core samples.

RNA isolation and 1st cDNA synthesis

Total RNA samples were prepared from eight selected *longissimus dorsi* animals for the gene expression analysis using Trizol reagent (Life Technologies Inc.) according to the manufacturer's instructions and quantified by absorbance at 260 nm. The first cDNA strand was synthesized from 3 μg of total

RNA pooled from four individual animals using the random hexamer primers (Promega Co.) and the superscript reverse transcriptase enzyme (Invitrogen Co).

Gene expression analysis by qRT-PCR

The gene expression of the selected 11 candidate genes was analyzed by real-time PCR. 18S rRNA gene (GenBank Acc No. AY779626) was used as an internal control. Realtime RT-PCR amplification mixtures (20 μl) contained 1 μl cDNA, 2 × SYBR Green I Master Mix (10 μl) (Qiagen., GmbH, Germany), and 10 pM forward and reverse primers. The real-time PCR reactions started at 95°C for 15 min for pre-denaturation, the reaction was then set at 95°C for 10 s, 56°C for 20 s and 72°C for 30 s, and 40 PCR cycles were performed. The PCR was conducted in ABI 7500 realtime PCR system (Applied Biosystems, USA). The relative gene expression level was calculated by the ΔCt method. The ΔCt value was determined by subtracting the 18S ribosomal Ct value from the target Ct value for each sample. The gene expression intensity was determined by calculating the $2^{-\Delta Ct}$.

Statistical analysis of qRT-PCR data

Statistical analysis was performed using an ANOVA model in an R statistical program (http://www.R-project.org). The gene expression intensity ($2^{-\Delta Ct}$) of 11 target genes was calculated by the ΔCt method, which was calculated by subtracting the 18S rRNA Ct value from Ct value of the target gene. As shown in Table 1, the factor, treatment (2 levels) and marbling score (2 levels; high (n = 4) is 16-32%; low (n = 4) is 4-7%) were fitted as fixed effects and the age of slaughter was fitted as a covariate in this model. The following statistical model was used to estimate the effect of the marbling group on individual gene expression.

$$Y_{ijk} = \mu + TR_i + FAT_j + bD_{ijk} + e_{ijk} \qquad [1]$$

Where Y_{ijk} is the target gene intensity ($2^{-\Delta Ct}$), μ is overall mean, TR_i is the fixed effect of i^{th} treatment, FAT_j is the fixed effect of j^{th} marbling score and D_{ijk} is the covariate term for the age of slaughter. Using this model, we examined the effect of the high- and low marbled groups on gene expression of the 11 candidate genes, and estimated their least square means (LSM) for testing if there was significant difference between the two groups using Student's t-test.

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