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# Identification of Single Nucleotide Polymorphism Marker and Association Analysis of Marbling Score in *Fas* Gene of Hanwoo

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**ABSTRACT:** The *Fas* (*APO-1*, *TNFRSF6*) gene known as a member of the tumor necrosis factor receptor superfamily was selected for DNA marker development in Korean cattle. It is a cell membrane protein and mediates programmed cell death (apoptosis). We discovered single nucleotide polymorphisms (SNPs) within *Fas* gene in order to develop novel DNA markers related to economical traits at the genomic level. The sequences of whole exon and 1 kb range of both front and back of the gene were determined by direct-sequencing methods using 24 cattle. A total of 55 SNPs were discovered and we selected 31 common polymorphic sites considering their allele frequencies, haplotype-tagging status and linkage disequilibrium (LD) for genotyping in larger-scale subjects. The SNPs were confirmed genotype through the SNaPshot method (n = 274) and were examined for a possible genetic association between Fas polymorphisms and marbling score. So, the SNPs that were identified significant are g.30256G>C, g.31474C>A, g.31940A>G, and g.32982G>A. These results suggest that SNPs of *Fas* gene were associated with intramuscular fat content of meat quality traits in Korean cattle. (**Key Words:** *Fas*, Hanwoo, Linkage Disequilibrium, Marbling Score, Single Nucleotide Polymorphism)

## INTRODUCTION

Marbling is one of the most important factors determining the grade of beef in the United States (USDA, 1989), Japan (JMGA, 1988), and South Korea, as well as having an effect on tenderness and juiciness. Therefore, various studies for improving intramuscular fat, such as extending period of fattening or high-energy feeding programs have been conducted in beef. However, these methods led to not only to the improvement of intramuscular fat but also to an increase of backfat and visceral fat in beef production. So, this undesirable consequence has progressed many studies to detect Quantitative trait loci (QTL) associated with economic traits for improvement of livestock through marker selection of superior cattle. This method selects DNA markers related to phenotype based on genetic value and statistical significance through association analysis between genetic variation and phenotype after identification of the genetic variation structure at each object. Then, the validated DNA marker can be used for Marker-Assisted Selection (MAS) (Lande and Thompson, 1990; Darvasi and Soller, 1994). In particular, single nucleotide polymorphisms (SNP) have been used to identify genetic markers for useful economic traits.

Direct sequencing of PCR (polymerase chain reaction) products have typically been applied to the identification of various gene mutations. However, with large numbers of samples, these approaches are time consuming and/or expensive. Primer extension assays have been developed for several genes with common mutations. SNaPshot is a commercially available kit for the multiplex detection of SNPs that relies on the extension of a primer annealed immediately adjacent to the SNP of interest, using fluorescently labeled dideoxynucleotides (ddNTPs). Each

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fluorescent ddNTP emits a different wavelength that is translated into a specific color for each base. The fluorescent labeled extension products can be visualized by electrophoresis using a capillary automated sequencer (Quintans et al., 2004).

*Fas* is a single polypeptide transmembrane protein that mediates apoptosis in a variety of hematopoietic, fibroblastic and malignant cells (Itoh et al., 1991; Oehm et al., 1992; Watanabe-Fukunaga et al., 1992). It was first recognized as an apoptosis inducer when activated human lymphocytes and certain cell lines underwent apoptosis upon exposure to monoclonal anti-Fas antibodies (Trauth et al., 1989; Yonehara et al., 1989). Molecular cloning of human and mouse Fas cDNAs demonstrated that it belongs to the tumor necrosis factor receptor (TNFR) superfamily, which includes the low-affinity nerve growth factor receptor (NGFR), TNFR types I and II, human B-cell antigen CD40, T-cell antigen CD27, and OX40 (Johnson et al., 1986; Stamenkovic et al., 1989; Loetscher et al., 1990; Mallett et al., 1990; Schall et al., 1990; Camerini et al., 1991).

In previous study, it found that Fas was negatively correlated to intramuscular fat content in the *m. longissimus* tissue of Korean cattle. HSPB1 expression in both mRNA and protein was shown to be negatively related to intramuscular fat content and was regulated by FAS and angiotensinogen. It suggests that the *Fas* gene may be one of the 258 key genes controlling adipogenesis through a mitogen-activated protein kinases signaling pathway. Therefore, Fas protein play an important role in the trait expression associated with fat synthesis (Kim et al., 2011). The objective of this study was to discover SNPs of the *Fas* gene located within QTL regions related to meat quality trait and to identify association with each SNP and marbling score (MS) traits in Hanwoo.

## MATERIALS AND METHODS

### Animals and phenotypes

Phenotype data and blood samples for SNP marker genotyping were obtained from 274 steers descending from 76 sires and unrelated dams from Hanwoo progeny-tested steers at Livestock Improvement Main Center. The Hanwoo received a total mixed diet of concentrate and rice straw with a ratio in total feed of about 1.5:1, 2:1, and 4.5:1 for growing period (4 to 12 months), finishing period I (13 to 18 months) and finishing period II (19 to 24 months), respectively for *ad libitum* intake. Crude protein and total digestible nutrients of the concentrate were 14%-16%, 11%-13% and 11% and 68% to 70%, 71% to 73%, and 72% to 73% for growing period, finishing period I and finishing period II, respectively. Phenotypic data in this study included carcass weight (CWT), eye muscle area (EMA), back fat thickness (BF) and MS. The BF, EMA, and MS were measured at the 12th to 13th rib junction after a 24 hour chill. The statistics for phenotypic data is summarized in Table 1. Marbling score was assessed on a 1 to 7 scale, and the degree of marbling was evaluated based on the Korean Beef Marbling Standard (BMS) from Animal Product Grading Service in Korea (APGS, 1995). Genomic DNA was separated from blood of cattle following modified salting out method (Miller et al., 1988). DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technology). DNA samples were diluted to 10 ng/μL and were stored at –20°C.

### Sequencing and SNP analysis

SNP identification in *Fas* gene was used for 24 different cattle having a different grandsire and sire. Primers for the sequence determination were produced to be about 700bp of PCR product using primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer3-www.results.cgiv) based on genetic information (BC140650.1) registered in the NCBI GenBank. Primers were selected for amplification of exon 7 (ex7-Fw 5'-CAAAATCAGGACAAATAGGCTTCT-3'; ex7-Rv 5'-TGTTGGAAAAAGATATGGTGACAG-3'), exon 8 (ex8-Fw 5'-AATTTGAATGGTGAAAGAATCCTC-3'; ex8-Rv 5'-TCTTATCCCAGCTCCTTCTATGTC-3') and exon 9 (ex9a-Fw 5'-GATAAGATGGTCATAAACCCTTGG-3'; ex9a-Rv 5'-AAGAAAACACACCCAGTAAAAAGC-3'; ex9b-Fw 5'-TCTTGCAGAGAAAATTTGTGACAT; ex9b-Rv 5'-CACCTGAGAAAGAAGTGGGTTATT-3'), with the amplicons covering position of 30,256, 31,474, 31,940, and 32,982.

PCR was performed in 20 μL volumes, each containing 50 ng of genomic DNA, 2 μL of 10× buffer (100 mM Tris pH 8.9, 50 mM KCl, 15 mM MgCl, 0.01% gelatin, 0.1% Triton X-100, 10 mg/mL bovine serum albumin), 0.5 μL of each primer (10 pmol), 1 μL of dNTPs (20 mM) and 0.5 unit of Hot Start Taq DNA polymerase (GeNet Bio, Daejeon, Korea). PCR conditions were 94°C for 5 min and 35 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, and a final step of 10 min at 72°C using a Tetrad 2 Peltier

**Table 1.** Means, standard deviation (SD) and extreme value of the phenotypes measured on carcass traits in Hanwoo

| Traits | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|
| LWT (kg) | 546.2 | 54.93 | 320.0 | 710.0 |
| CWT (kg) | 321.7 | 34.45 | 174.0 | 423.0 |
| DP(%) | 58.89 | 2.081 | 54.18 | 58.89 |
| EMA ($cm^2$) | 74.20 | 8.569 | 30.00 | 99.00 |
| BF (cm) | 8.161 | 3.041 | 3.000 | 21.00 |
| MS (1-7) | 1.814 | 1.099 | 1.000 | 7.000 |

LWT, live weight; CWT, carcass weight; DP, dressing percentage; EMA, eye muscle area; BF, backfat thickness; MS, marbling score.

Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR product identified on 1.5% agarose gel was filtered by MultiScreen filter plates (Millipore, Billerica, MA, USA). Then PCR was performed in 10 μL volumes, each containing 2 μL of Filtered PCR product, 1.75 μL of 5× buffer (400 mM tris, 10 mM $MgCl_2$), 0.5 μL of Big Dye and 1 μL of each primer (5 pmol). PCR conditions were 96°C for 10 s and 34 cycles of 5 s at 50°C, 4 min at 60°C. PCR product was refined using alcohol after PCR and dried. Dried sample was added with 8 μL of Hi-Di Formamide (Applied Biosystems, Waltham, MA, USA) and allowed to react for 2 min at 95°C. Then, DNA sequence was analyzed on ABI 3730XL Genetic Analyzer (Applied Biosystems, USA). SNP was analyzed by the Lasergene software (Version 7, DNA Star, Madison, WI, USA).

**SNaPshot and genotyping**

SNaPshot probes were manually designed by checking their annealing temperature (Table 2). The length of a primer was modified by the addition of non-homologous polynucleotides at the 5' end. The primers for PCR and SNaPshot analysis were synthesized by Bioneers (Daejeon, Korea). The SNaPshot reaction was performed in a total volume of 5 μL using 2 μL of SNaPshot Mastermix, 2 μL of primer and 1 μL of filtered PCR product. Thermal cycling conditions were 25 cycles of 10 s at 96°C, 5 s at 55°C and 30 s at 60°C. The SNaPshot products were then cleaned by adding 1 μL of Shrimph alkaline phosphatase (SAP, Thermo Fisher, Waltham, MA, USA) and 1 h incubation at 37°C, followed by a 15 min enzyme denaturation at 75°C. Fragments were separated on an ABI 3730-Genetic Analyzer (Applied Biosystems) using 8.5 μL of formamide, 0.5 μL of GeneScan-120 LIZ size standard (Applied Biosystems) and 1 μL of cleaned SNaPshot product. A 36 cm capillary array and the polymer POP-4 were used and the instrument settings were defaulted as recommended by the manufacturer. Raw data was analyzed using the Lasergene software (DNA Star, USA). Main data analysis was accomplished with a PHASE v2.1.1 and Haploxt software (University of Michigan, Ann Arbor, MI, USA).

**Statistical analyses**

Analysis of the statistical relevance with the phenotypic measurement and the four SNP genotype of *Fas* gene were evaluated with the mixed regression models using ASReml 3.0 program (Gilmour et al., 2006). Also, p-value less than 0.01 were accepted as statistically significant. The difference of mean value was performed a test of significance of difference of genotype through a Fisher's least significant difference test using the following statistical linear model:

$$Y_{ijk} = \mu + YS_i + b \cdot D_{ijk} + Genotype_j + A_{ijk} + e_{ijk}$$

where $Y_{ijk}$ is the observation of the carcass traits, $\mu$ is the overall mean for each trait, $YS_i$ is the effect of season, $b$ is convariation of slaughter age, $D_{ijk}$ is slaughter age (month), $Genotype_j$ is the effect of genotype, $A_{ijk}$ is the fixed effect of individual and $e_{ijk}$ is the random residual effect.

## RESULTS AND DISCUSSION

Marbling is generally considered to increase juiciness and tenderness of meat, and therefore to have a positive association with eating quality (Wood et al., 1995; Gutierrez-Gil et al., 2008). The marbling increase is an economically important trait of beef industry in Korea. A better knowledge of the molecular mechanism of marbling is important as it may generate new opportunities for more effective MAS, leading to economic benefits to the beef industry (Yamada et al., 2009). Recently, there have been many efforts to improve meat quality following the development of genomics using DNA markers in livestock (Maj et al., 2006; Otto et al., 2007; Gill et al., 2010).

The *Fas* gene, a member of the (TNF) receptor superfamily that mediates apoptosis in a various cells, was known to associate with intramuscular fat content from previous study (Kim et al., 2011). *Fas* gene has nine exons and is located at bovine chromosome 26 (BTA26) from AC_000183.1 (10714194..10748026, Bos _taurus_UMD_3.1). The sequences of whole exon and 1kb range of both front and back of the gene were determined by PCR-direct sequencing using 24 different cattle each having a different grandsire and sire. The homology was more than 99% with sequence reported in GenBank (BC140650.1). It was selected SNP of locus having mutation frequency of 0.01 or more and nucleotide both homo- and hetero-type. The 55 SNPs were discovered. And, 33 polymorphic sites were selected considering their allele frequencies, haplotype-

**Table 2.** SNaPshot probes for the detection of *Fas* genotypes

| Probe | Sequence(5' → 3') | Position | SNP | Size (bp) |
|---|---|---|---|---|
| SNP1 | $at_{(10)}$CCCCCTACTTGTTACTTTTTAAAAAGCAAAC | g.30256 | G>C | 51 |
| SNP2 | CTGAATTGTAGACTCAGTTGTATACTTACTC | g.31474 | C>A | 31 |
| SNP3 | $ta_{(8)}$tAAAAGTGGGAATTTTGTTTAGAAAAACAAATTTTCAGA | g.31940 | A>G | 55 |
| SNP4 | $ta_{(8)}$tCAGACTCAGTTATAATGCTTGAATATTTTATATTTGTC | g.32982 | G>A | 55 |

SNP, single nucleotide polymorphism.

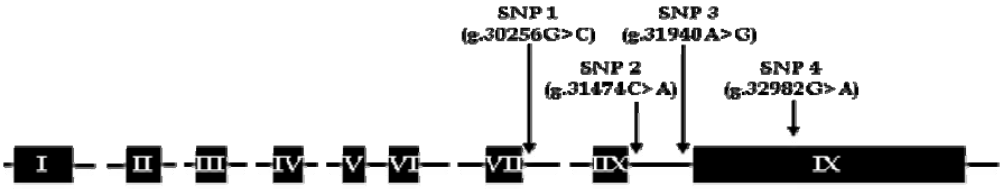


**Figure 1.** The positions of detected SNPs at tumor necrosis factor receptor superfamily member 6 (*Fas*) gene in Korean cattle (Hanwoo). SNPs, single nucleotide polymorphisms.

tagging status and linkage disequilibrium (LD) for genotyping of larger-scale subjects. The SNPs were confirmed genotype through SNaPshot method using 274 Hanwoo progeny-tested steers at Livestock Improvement Main Center and were examined for possible genetic association of Fas polymorphisms with MS. As a result, single locus association analysis for *Fas* gene found that four SNPs were associated with the meat quality trait. The SNPs in intron VII and IIX region and in exon IX region was identified (Figure 1). The four SNPs was verified by the dbSNP of GenBank. It was showed to be significant SNP of g.30256G>C, g.31474C>A, g.31940A>G, and g.32982G>A with MS ($p<0.001$) (Table 3). In case of g.30256G>C SNP identified significant association, the GC genotype was shown to be superior to the more than CC and GG genotypes. Similarly, g.31940A>G and g.32928G>A was shown to be superior to the GA genotype more than GG and AA genotypes. But at locus g.31474C>A, Hanwoo with homozygous genotype AA showed a significantly higher marbling score compared with those of heterozygous genotype CA or homozygous genotype CC. The results give strong evidence for the potential of MAS for marbling and could produce substantial improvement of meat quality traits in Hanwoo.

The three SNPs were located in intron region and one SNP was located in exon region. The intron SNPs do not regulate the function of the gene. Generally, introns are considered superfluous parts that must be removed to make a mature mRNA. However, it is known that they are facilitators of molecular evolution and regulators of gene expression. It was demonstrated in several systems, including mammalian tissue culture cells, transgenic mice, insects, and plants, that optimal expression of many endogenous genes similarly requires the presence of one or more introns (Callis et al., 1987; Buchman and Berg, 1988; Chiou et al., 1991; Palmiter et al., 1991; Duncker et al., 1997; Bourdon et al., 2001). And the nucleotide variation of introns in specific genes plays an important role in transcription and translation (Nott et al., 2003). The magnitude of intron-dependent effects can vary tremendously, from almost nothing to more than a 400-fold increase in mRNA levels (Buchman and Berg, 1988; Bourdon et al., 2001).

Chromosomes such as BTA14 and BTA26 known to have exon regions linked to meat quality trait QTL are primary sites for the presence of functionally important genes affecting lipid metabolism (Casas et al., 2004; Marques et al., 2009). Especially, association studies of economic traits and SNPs have implicated many genes in the BTA26 QTL region (Taniguchi et al., 2004; Gautier et al., 2006). Therefore, it could be a LD effect with lipid metabolism-related genes associated with the QTL of each trait presenting around the *Fas* gene. A previous study reported that *Fas* gene was negatively correlated to intramuscular fat content (Kim et al., 2011). The *Fas* gene was thought to have an association with economic traits. The QTL associated with these traits may be LD markers. The bovine *Fas* gene in the BTA26 region appeared to be related to MS directly or indirectly or both in Hanwoo.

In summary, we identified 55 SNPs and selected 33 SNPs through haplotype-tagging status and LD analysis in Hanwoo *Fas* gene. Genotyping analysis and association analysis were performed on selected SNPs. Four SNPs were significantly associated with MS in economic traits. The meat quality grade in beef uses the BF, EMA, CWT, and MS having significant impact in quality of the cattle carcass.

**Table 3.** Least square means and standard errors of phenotypic measurements from carcass traits by *Fas* genotypes in Hanwoo

| Trait | Type | Genotype | | | p value |
|---|---|---|---|---|---|
| Marbling score | g.30256G>C | CC | GC | GG | p<0.001 |
| | | 1.77±0.15 | 2.08±0.13 | 1.70±0.13 | |
| | g.31474C>A | AA | CA | CC | p<0.001 |
| | | 2.02±0.12 | 1.51±0.27 | 1.676±0.17 | |
| | g.31940A>G | GG | GA | AA | p<0.001 |
| | | 1.77±0.14 | 2.04±0.12 | 1.65±0.13 | |
| | g.32982G>A | AA | GA | GG | p<0.001 |
| | | 1.68±0.16 | 2.04±0.12 | 1.68±0.13 | |

Therefore, it was considered that the *Fas* gene could be used to improve the selection of Hanwoo. The goal of this study was to find information about molecular markers for MAS. The identification of the causative mutations accounting for the variability at these economic traits would be not only allow increased accuracy of selection but also allow focused genotyping of essential markers for selection of a specific trait. Further study will be necessary in larger populations to validate the use these SNP for MAS. It is also important to investigate whether the *Fas* gene plays a role in the development of these traits and whether it is involved in LD with other causative mutations.

## CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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