Association between an electron transfer flavoprotein alpha subunit polymorphism (rs321948383) and the meat quality of Berkshire pigs

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ABSTRACT
The electron transfer flavoprotein α subunit gene (ETFA) encodes a protein that forms part of the ETF enzyme. This enzyme is normally active in mitochondria, the energy-producing centres of cells, to donate electrons derived from fatty acid oxidation to ETF- ubiquinone oxidoreductase. In the present study, we identified a non-synonymous single-nucleotide polymorphism (nsSNP) (rs321948383, c.569G > A) in the porcine ETFA gene and analyzed the association between this nsSNP and meat quality traits in 405 Berkshire pigs. The pigs with rs321948383 G/G had a higher meat quality from the aspects of meat colour (L*, p = 0.013), water-holding capacity (p = 0.001), drip loss (p = 0.000), carcass temperatures (T1, p = 0.000; T4, p = 0.000; T12, p = 0.000; T24, p = 0.001), and pH24 (p = 0.014). Therefore, this nsSNP (c.569G > A) could play an important role in improving the meat quality of pigs.

Introduction
The electron transfer flavoprotein (ETF) participates in catalysis of the initial step of mitochondrial fatty acid β-oxidation, as well as oxidation of certain amino acids (Ikeda and Tanaka 1983; Freeman 1988; Eder et al. 1997; Watmough and Freeman 2010). The protein is heterodimeric: the α and β subunits exhibit considerable sequence and structural homologies (Roberts et al. 1996). The ETF α subunit (ETFα) is synthesised in the cytosol as a 35-kDa precursor that is imported into the mitochondrial matrix and processed to the 32-kDa mature form via proteolytic cleavage. In contrast, the 27-kDa ETF β-subunit (ETFβ) is not processed after import into mitochondria (Ikeda et al. 1986). Both chains shuttle electrons between the primary flavoprotein dehydrogenases involved in mitochondrial fatty acid and amino acid catabolism and the membrane-bound ETF-ubiquinone oxidoreductase.

Lipid oxidation influences meat quality in a number of ways, including off-flavor formation, drip loss, and colour changes. During lipid oxidation, polyunsaturated fatty acids are degraded to volatile short-chain oxidation products, which trigger off-odors and off-flavors. Such oxidation is strongly enhanced during meat cooking and storage. The formation of volatile lipid oxidation products strongly reduces product acceptability. Oxidative processes can also affect the ability of membranes to hold water and may contribute to drip loss (Jensen et al. 1998; Weber and Antipatis 2001). Moreover, several studies have shown that lipid oxidation is the most common form of chemical damage to meat (Medina-Meza et al. 2014; Shah et al. 2014).

In this study, we identified a single-nucleotide polymorphism (SNP) of the ETFα gene and evaluated the associations between this polymorphism and the meat quality traits of Berkshire pigs.

Materials and methods
Ethics statement
Animal care and use, and all experiments were carried out in accordance with the guidelines of the Gyeongnam National University of Science and Technology Institutional Animal Care and Use Committee.

Animals and meat quality traits
A total of 405 purebred Berkshire pigs were examined in this study. They were all reared at the same pig farm which is a large scale farm with over 1000 sows (Dasan Pig Breeding Co., Namwon, Korea), randomly selected, and slaughtered at body weights of 110 ± 10 kg in 10 batches. Each batch had approximately 40 pigs and all animals were held in the lairage for 8 h pre-slaughter without feed and water. After slaughter, the carcass weight (kg) was recorded, and backfat thickness (mm) was measured at the 10th rib at a distance three-quarters down the longissimus dorsi toward the belly. To measure meat quality traits, the longissimus muscle samples were collected immediately after slaughter and maintained at 4°C. The meat quality traits consisted of meat colours (L* [light-
ness), a* [redness], and b* [yellowness]); water-holding capacity (%); drip loss (%); cooking loss (%); Warner-Bratzler shear force (kg/cm²); chemical composition (moisture, collagen, crude protein, and crude fat; all %); carcass temperatures (°C) at 1, 4, and 12 h after slaughter (T1, T4, and T12, respectively); and the 24-h postmortem pH (pH24). Meat colours were determined in the fresh cross-section of longissimus dorsi 24 h after slaughter using a Minolta Chromameter (CR-400; Minolta, Osaka, Japan) after 30 min of blooming at 1°C. Water-holding capacity was measured at 3 days postmortem using a centrifugation method. Duplicate 10-g minced samples taken from one chop from each loin were placed into centrifuge tubes and spun for 10 min at 40,000 g. After centrifugation, the liquid was removed and the meat re-weighed. The percentage of water loss was calculated and used to estimate the water-holding capacity. Drip loss was calculated as the weight difference before and after storage. A slice 2 cm in thickness (weight 100 ± 5 g) taken from the longissimus muscle was placed in a polypropylene bag (Dongbang Co., Gimpo, Korea), vacuum-packed, and then stored for 24 h at 4°C. After centrifugation, the liquid was removed and the meat re-weighed. The percentage of water loss was calculated and used to estimate the water-holding capacity. Drip loss was calculated as the weight difference before and after storage. A slice 2 cm in thickness (weight 100 ± 5 g) taken from the longissimus muscle was placed in a polypropylene bag (Dongbang Co., Gimpo, Korea), vacuum-packed, and then stored for 24 h at 4°C. After centrifugation, the liquid was removed and the meat re-weighed. The percentage of water loss was calculated and used to estimate the water-holding capacity. Drip loss was calculated as the weight difference before and after storage. 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**Tissue expression of the ETFA gene**

Semiquantitative RT–PCR was performed to determine the expression profile of the ETFA gene in various tissues of Berkshire pigs. Total RNAs from various tissues (liver, stomach, lungs, kidney, large and small intestines, spleen, and muscle) of three Berkshire pigs were isolated using the TRIzol- Reagent (Molecular Research Center, Cincinnati, OH, USA) and reverse-transcribed into cDNAs with the aid of Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocol. The cDNAs were then subjected to PCR for evaluation of the relative gene expression level of ETFA and that of the gene encoding peptidylprolyl isomerase A (PPIA) (internal control), using appropriate primer

![Figure 1](image-url)  
*Figure 1.* The porcine ETFA mRNA expression was determined in various tissues by RT-PCR. RNA was isolated from various tissues, including the liver, stomach, lung, kidney, large and small intestines, spleen and muscle. Values are mean ± SD.
pairs (Table 1). Amplification reactions were performed on a Perkin Elmer 9700 system (Applied Biosystems, Waltham, MA, USA) under the following conditions: 95°C for 5 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and final elongation for 7 min at 72°C. The PCR products were separated on 2% (w/v) agarose gels and quantified using a Gel Logic model 200 imaging system (Kodak, Rochester, NY, USA).

**ETFA SNP detection and genotyping**

mRNAs were isolated from pooled liver tissues of three Berkshire pigs using an RNA-sequencing (RNA-seq) sample preparation kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer’s instructions, and an ETFA SNP was identified in cDNAs synthesised from the RNA templates using an Illumina GAII analyzer (Illumina, Inc., San Diego, CA, USA) on the basis of comparisons made with the NCBI dbSNP database, as described previously (Jung et al. 2012). To examine the genotypes of the ETFA SNP, genomic DNAs were isolated from whole blood cells of all 405 pigs and SNP genotypes were analyzed using the Illumina VeraCode GoldenGate Assay kit (Illumina, Inc.). Table 1 lists the oligonucleotides used for the genotyping of ETFA SNPs.

**Statistical analysis**

The associations between ETFA genotypes and meat quality traits were evaluated using a general linear model employing SAS software version 9.13 (SAS Institute Inc., Cary, NC, USA). SNPs subjected to statistical analysis were characterised by a call rate <0.90, a minor allele frequency >0.01, and a Hardy-Weinberg equilibrium (p value) >0.05. The linear model employed was:

\[ y_{ij} = \mu + G_i + S_j + e_{ij} \]

where \( y_{ij} \) is the phenotypic contribution of the target trait, \( \mu \) is the general mean, \( G_i \) is the fixed effect of genotype \( i \), \( S_j \) is the fixed effect of sex \( j \), and \( e_{ij} \) is the random error. ANOVA with the Bonferroni correction and the Kruskal-Wallis test were used to verify significant differences (p < 0.05) between the genotypic frequencies of traits.

**Results**

**Tissue expression of the ETFA gene**

The tissue expression pattern of ETFA mRNA was examined in various Berkshire pig tissues with the aid of semiquantitative RT–PCR (Figure 1). The PPIA gene served as a reference gene (Park et al. 2015). ETFA was ubiquitously expressed in all tissues tested; no significant between-tissue difference was apparent. However, the expression levels were relatively higher in muscle, kidney, and liver than in other tissues.

**Identification of an ETFA SNP and genotyping**

RNA-seq was used to discover a non-synonymous SNP (nsSNP; rs321948383, c.569G > A) in porcine ETFA in liver tissue. This nsSNP changed serine to asparagine at position 190. To analyze the associations between this nsSNP and meat quality, we genotyped 405 Berkshire pigs using the GoldenGate assay. All three genotypes (GG, GA, and AA) were detected in the pig population. Table 2 lists detailed information on the SNP as well as the allele frequencies. The GG genotype was much more common than the GA and AA genotypes. The frequencies of the G and A alleles were 0.910 and 0.090, respectively. The genotype frequencies were in Hardy-Weinberg equilibrium (p > 0.05) (Falcofer 1996). Nevertheless, we excluded AA genotype in the association analysis, since the number of the pigs with it is negligible.

**Association between ETFA nsSNP and meat quality**

The nsSNP of porcine ETFA was investigated in terms of associations with the meat quality traits of 405 Berkshire pigs. As...
shown in Table 3, the nsSNP was significantly associated with lightness (the CIE L* value, $p = 0.013$), water-holding capacity ($p = 0.001$), drip loss ($p = 0.000$), carcass temperatures (T1 ($p = 0.005$), T4 ($p = 0.000$), T12 ($p = 0.000$), and T24 ($p = 0.001$)), and postmortem $pH_{24}$ ($p = 0.014$). The GG genotype was associated with higher meat quality than the GA genotype.

**Discussion**

In this study, porcine *ETFA*, which is normally active in the mitochondria (the energy-producing centres of cells), was expressed at relatively higher levels in muscle, liver, and kidney than in other tissues. Metabolism of glucose and fat after slaughter converts muscle to meat, and these factors are influenced by muscle composition, which thus affects the final meat quality (Wenk et al. 2000; Shen et al. 2014). Fatty acids can be used to generate energy or can be stored in the body for later oxidation. Skeletal muscle is the main site of fatty acid oxidation (Jeukendrup 2002). *ETFA*, which is involved in mitochondrial fatty acid β-oxidation, is a crucial player in cellular energy transduction, particularly in organs such as muscles that rely on fatty acids for energy. Therefore, we assumed that *ETFA* status would be a determinant of meat quality.

We found evidence for an association between *ETFA* and meat quality traits in Berkshire pigs. The nsSNP rs321948383; c.569G > A, located in the *ETFA* gene, significantly affected lightness (the CIE L* value), water-holding capacity, drip loss, carcass temperatures (T1, T4, T12, and T24), and postmortem $pH_{24}$. The GG genotype had a higher pH and lower temperatures than other genotype. The combination of low pH and high temperature results in muscle protein denaturation which leads to the reduced water holding capacity. Thus, pigs with the homozygous GG genotype exhibited significantly higher meat quality than did those with the GA genotype.

*ETFA* is located in the mitochondrial matrix, and the ETF combination (*ETFA* and *ETFB*) accepts electrons from at least nine different acyl-CoA dehydrogenases. These electrons are transferred to the electron transport chain via a membrane-bound iron-sulfur flavoprotein, ETF–ubiquinone oxidoreductase (Ikeda and Tanaka 1983; Indo et al. 1992; Ghisla and Thorpe 2004; Schiødt et al. 2006). This process is central to β-oxidation, which provides energy to the organism. In live animals, continuous control of enzymatic β-oxidation is evident. However, after slaughter, the cellular mechanism controlling lipid oxidation is now absent and lipid peroxidation may develop. Lipid hydroperoxides, postmortem primary products of lipid decomposition, form volatiles including alkanes, aldehydes, and alcohols that may be toxic to cells (Toldra 2017). ETF produces significant amounts of hydrogen peroxide, which is a precursor to other peroxides when acted upon by the partner enzyme medium-chain acyl-CoA dehydrogenase (Acton 2013). Furthermore, early after slaughter, oxidative status (the extent of fatty acid oxidation) can be influenced by muscle pH and temperature (Toldra 2017), suggesting that *ETFA* status may affect meat quality by regulating fatty acid oxidation.

In summary, we explored the association between an *ETFA* polymorphism and meat quality in the Berkshire pig. The *ETFA* GG genotype improved meat colour, water-holding capacity, drip loss, carcass temperatures, and postmortem $pH_{24}$. Therefore, this nsSNP could play an important role in improving the meat quality of pigs. However, to complete the potential of the gene as genetic marker, further studies on different large populations are needed.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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