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IDENTIFICATION OF A SYNONYMOUS SNP IN THE FTL GENE AND ITS ASSOCIATION WITH MEAT QUALITY TRAITS IN BERKSHIRE PIGS

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ABSTRACT

Ferritin light chain (*FTL*) is a subunit of the ferrit in protein, which plays an essential role in intracellular iron storage. To evaluate the association of *FTL* gene expression and meat quality, the expression patterns of *FTL* in four pig breeds, Berkshire, Duroc, Landrace, and Yorkshire, were examined. The *FTL* mRNA expression in liver and kidney of all four breeds was noticeably higher when we compared to other tissues examined in this study. To identify novel single nucleotide polymorphisms (SNPs) in the *FTL* gene, the livers of Berkshire pigs were examined and a synonymous SNP, *FTL* c.472*C*>*G* Pro, was identified. In the same population, various meat quality traits were evaluated and the relationship between *FTL* genotype and meat quality was examined. The identified *FTL* SNP was significantly associated with carcass weight, meat color, drip loss, water holding capacity, post-mortem pH_{24hr}, and chemical composition (collagen and fat content). The *C* allele showed higher carcass weight, water holding capacity, and post-mortem $pH_{24hr}$and lower drip loss which considered higher economic merit and meat quality. Together, these data suggest that *FTL* synonymous SNPs may be an important factor for determining meat quality in Berkshire pigs.

INTRODUCTION

Meat quality is affected by various factors, including genetics and the environment. Molecular genetics approaches have generated novel methods to discover the genomic regions responsible for determining meat quality. In particular, SNPs in specific genes are suggested to be predictive for carcass traits and meat quality (Barendse, Bunch, and Harrison 2005; Buchanan et al. 2002; Page et al. 2002; Lee et al. 2011; Cho, Ha, et al. 2015; Cho, Lee, et al. 2015). Ferritin is an iron binding protein consisting of 24 subunits made up of heavy and light polypeptide chains of about 21,000 and 19,000 Daltons, respectively (Boyd et al. 1985). The two polypeptide chains have highly similar sequences, but each originate from different gene families (Jain et al. 1985).

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The ratio of heavy to light subunits in ferritin is diverse, and depends on the tissue type and physiological state of the cell. Heavy subunits dominate in the heart and kidney, whereas light subunits are more commonly found in the liver and spleen (Torti and Torti 2002). Ferritin light chain (*FTL*), which is shorter than ferritin heavy chain (*FTH*) by eight amino acids, plays a role in lowering the concentration of *FTH*, and is more resistant to denaturing agents and temperature (Goralska, Holley, and McGahan 2003; Muhoberac and Vidal 2013). Complete sequences of *FTL* from horse spleen and human liver and spleen have been determined through amino acid sequencing (Heusterspreute and Crichton 1981; Wustefeld and Crichton 1982; Addison et al. 1983). Ferritin plays a critical role in cellular and organismal iron homeostasis by capturing and buffering the intracellular iron pool. Because of the toxicity of free iron, the role of ferritin is physiologically important (Muhoberac and Vidal 2013). Lipid peroxidation is the main reason for quality deterioration in meat and meat products (National Research Council, 1988). Lipid peroxidation changes the flavor, color, texture, and drip loss of the meat.

Importantly, it also decreases its nutrition value and functionality, and generates harmful compounds (Addis 1986; Kanner 1994; Morrissey et al. 1998). Oxygen is the most important factor for the progress of lipid peroxidation in meat (Ahn et al. 1992; Ahn et al. 1993). Although ground state oxygen is not directly involved in lipid peroxidation, reactive oxygen species (ROS) are highly reactive and can initiate lipid peroxidation (Halliwell and Gutteridge 1999). Interestingly, ferritin has been shown to protect cells from oxidation and lipid peroxidation after slaughter (Decker and Welch 1990; Kanner and Doll 1991; Han et al. 1993). In this study, the expression pattern of *FTL* in various tissues was examined in four different pig breeds. *FTL* gene SNPs were then investigated and their association with meat quality in Berkshire pigs was determined. In all, we found a novel SNP in the *FTL* gene and showed its association with meat quality in Berkshire pigs.

MATERIALS AND METHODS

Animals

A total of 416 pigs from the Baekdusan pedigree line (males = 200, females = 216, slaughtered in batches of 10), a new pedigree line originating from American Berkshire pigs and registered with the Korean Animal Improvement Association (KAIA) in 2008, were chosen for this study to analyze SNP in *FTL* gene and meat quality traits. The pigs were reared under the same environmental conditions and randomly selected for slaughter when their body weights reached 110 kg. To test carcass traits, the Berkshire pigs were slaughtered by stunning with electrical tongs (300 volts for 3 s) after 12 h of feed restriction. After the stunned pigs were exsanguinated, carcasses were dehaired at 62°C for 5 min, and any remaining hair was removed using a knife and flame. Carcasses were eviscerated and split before being placed in a chiller set at 2~4°C for 12 h.To compare the mRNA expression pattern of FTL through the tissues, we prepared various tissues including liver, stomach, lung, kidney, large intestine, small intestine, and spleen of tree pigs from each pig breed such as Berkshire, Landrace, Yorkshire, and Duroc.

RNA-Sequencing

The liver tissue of Berkshire was used for RNA-Seq. mRNA was isolated and purified by using an RNA-Seq sample preparation kit (Illumina, Inc., San Diego, CA, USA; (Jung et al. 2012)). The quality of total RNA was measured by Nanodrop (Thermo Scientific, Wilmington, DE, USA). All extractions exhibited an RNA integrity number >8.0. Cleaved RNA fragments primed with random hexamers were reverse transcribed into first strand cDNA using reverse transcriptase and random primers. RNA template was removed and synthesized a replacement strand to generate double strand cDNA. End pairing, A-tailing, adaptor ligation, cDNA template purification and enrichment were performed. We developed final RNA-Seq library and performed quality check using Bioanalyzer. The completed RNA library was performed paired-end 100bps sequencing using Hiseq2000 (Illumina, Inc., San Diego, CA, USA).

SNP analysis

SNP was analyzed using UCSC Pig genome (SGSC Sscrofa10.2/susScr3) by mapping sequence from the result of RNA-Seq. The standard SNP discovery was determined by detection of mutation within read coverage 30 or more. The analysis of SNP was performed by snpEffsoftward.

RT-qPCR amplification

Total 2 μgRNA from liver, stomach, lung, kidney, large intestine, small intestine, and spleen of four pig breeds: Berkshire, Duroc, Landrace, and Yorkshirewas isolated using TRI-reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions and then was used for reverse transcription in a volume of 20 μl. RT-qPCR was performed using a SYBR Green by Rotor Gene Q thermocycler (Qiagen, Hilden, Germany) as previously described (Park, Kwon, Hwang, Park da, et al. 2015). Briefly, the Primer3 program was used to design *FTL* gene specific primers (Table 1). The PCR reaction was cycled at 94°C for 5 s followed by 60°C for 10 s for 40 cycles. The total amount in the PCR reaction mix was: 20 μL [1 μL cDNA, 10 μL Rotor Gene SYBR Green PCR mastermix, 1 μl each of the forward and reverse primers (10 pmol), and 7 μL H_2O]. The melting curve was analyzed to confirm the amplification specificity of each RT-qPCR reaction. The temperature range for analysis of the melting curves was 70°C to 95°C for 5 s. Three independent experiments were performed for data analysis. The fold change of FTL mRNA was analyzed by the relative quantification method derived from the delta-Cq geometric means after normalization with reference genes (*PPIA*, *TBP*,and *HSPCB* in Berkshire pigs, *PPIA*, *TBP*, *RPL4*, and *RPS18* in Landrace pigs, *PPIA* and *TBP* in Duroc pigs, and *PPIA*, *TOP2B*, *RPL4*, and *RPS18* in Yorkshire pigs)(Park, Kwon, Hwang, Park, et al. 2015).

Analysis of *FTL* genotype

Genomic DNA was isolated using individual *longissimus dorsi* muscles from Berkshire pigs using Wizard Genomic DNA Purification kit (Promega, Madison, USA). To analyze the association between *FTL* SNPs and pork meat quality traits, gDNA from the *longissimus dorsi* muscles of 416 Berkshire pigs were examined using a VeracodeGoldenGate assay kit (Ilumina, San Diego, CA, USA). The analyzed oligonucleotides are shown in Table 1.

Analysis of meat quality traits

In total, 416 Berkshire pigs were used for meat quality analysis and genotyping of SNP in *FTL* gene as *CC*, *CG*, and *GG*types. Backfat from the 10th rib region three-quarters along the *longissimus dorsi* toward the belly was used to measure backfat thickness. The *longissimus dorsi* (6^{th} to 13^{th} rib) was excised and maintained at 2~4°C before it was transported to the laboratory for chemical composition determination. The moisture, crude protein (CP), and crude fat (CF) concentrations of the *longissimus dorsi*muscles were examined following the methods outlined by the Association of Official Agricultural Chemists(Chemists 1995). The measurement of water holding capacity was determined by centrifugation method as previously described (Kristensen and Purslow 2001). The meat lightness and fat lightness were recorded after 30 min at 1℃ using a Minolta Chromameter (CR400; Minolta, Japan). The light source (illuminat C 2° observer) was standardized to a white standard plate (L* = +97.83, a* =-0.43, b* = +1.98). For drip loss measurement, a *longissimusdorsi* muscleslice of 2 cm thickness (weight 100±5 g) was placed

into a polypropylene bag (Oxygen Transmission, Dongbang Co., Korea) packaged by vacuum and stored for 24 h at 4℃. The weight difference before and after 24 h was calculated as drip loss. For measurement of cooking loss, a slice of 3 cm thick *longissimus dorsi* muscle (weight 100±5 g) was packed into a polypropylene bag (Oxygen Transmission, Dongbang Co., Korea), cooked for 40 min at 70℃ in a water-bath, and then cooled to room temperature. Cooking loss was calculated by determining the weight difference before and after cooking. Collagen content was measured using 4 g of *logissimusdorsi* muscle put into a triangular flask, mixed with 30 ml of 7 N sulfuric acid, and then heated in a dry oven at 105℃ for 16 h. After homogenization, the fluid was filtered using Whatman No. 2 150 mm filter paper.

Collagen content (g/100 g) was calculated by the regression equation, and the standard curve was obtained by measuring the color reagent and working standard solution (0.6, 1.2, 1.8, and 2.4 μg hydroxyproline/mL).

Statistical analysis

The general linear model procedure was used to analyze the association between genotypes and traits using Statistical Software Package version 9.1.3 (Guide 2004). The linear model was: $y_{ijklm} = \mu + G_i + S_i + P_l + e_{ijklm}$, where y_{ijklm} was the phenotypic value of the target trait, μ was the general mean, G_i was the fixed effect of genotype i, S_j was the fixed effect of sex j, P_l was the fixed effect of the slaughter period l, and e_{ijklm} was the random error.

Table S1. Analyses of association between meat quality traitsand FTL synonymous SNP*c.472C>G*

Model	Dominant			Recessive		
Genotype	GG+CG (n=412)	CC (n=4)	P value	GG (n=285)	CG+CC (n=131)	P value
Backfat thickness	24.993±0.255	21.750±3.092	0.2401, ns	25.109±0.300	24.638±0.475	0.6867, ns
CIE *L*	48.621±0.142	48.643±0.427	0.9750, ns	48.704±0.174	48.440±0.238	0.3974, ns
Moisture	75.531±0.041	76.168±0.389	0.1202, ns	75.535±0.049	75.540±0.072	0.9421, ns
Protein	23.837±0.036	24.051±0.124	0.4723, ns	23.818±0.044	23.884±0.063	0.3277, ns
Cooking loss	27.239±0.184	25.653±1.760	0.3525, ns	27.495±0.213	26.629±0.350	0.094, ns
Warner-Bratzler shear force (kg)	2.887±0.033	2.922±0.349	0.7935, ns	2.937±0.041	2.780±0.056	0.059, ns
Post-mortem pH_{45min} B	6.304±0.016	6.240±0.017	0.6344, ns	6.308±0.020	6.292±0.025	0.7526, ns
Post-mortem pH_{45min} L	6.007±0.013	6.038±0.176	0.6353, ns	6.001±0.016	6.021±0.025	0.5520, ns

Model	Codominant						
Genotype	CC (n=4)	CG (n=127)	GG (n=285)	P value	P value in CC vs. CG	P value in CC vs. GG	P value in CG vs. GG
Carcass weight (kg)	92.000±1.732	85.732±0.446	85.779±0.350	0.0673	P>0.05, ns	P>0.05, ns	P>0.05, ns
Backfat thickness(mm)	21.750±3.092	24.730±0.483	25.109±0.300	0.4853	P>0.05, ns	P>0.05, ns	P>0.05, ns
Post-mortem Ph_{24hr}	6.036±0.088	5.822±0.019	5.812±0.012	0.086	P>0.05, ns	P>0.05, ns	P>0.05, ns
CIE *L*	48.643±0.427	48.433±0.245	48.704±0.174	0.6959	P>0.05, ns	P>0.05, ns	P>0.05, ns
CIE *b*	3.163±0.379	2.853±0.090	2.881±0.068	0.7738	P>0.05, ns	P>0.05, ns	P>0.05, ns
Collagen(%)	0.761±0.006	0.881±0.011	0.893±0.008	0.1028	P>0.05, ns	P>0.05, ns	P>0.05, ns
Fat(%)	2.251±0.324	2.776±0.096	2.813±0.069	0.6217	P>0.05, ns	P>0.05, ns	P>0.05, ns
Moisture(%)	76.168±0.389	75.520±0.073	75.535±0.049	0.2846	P>0.05, ns	P>0.05, ns	P>0.05, ns
Protein(%)	24.051±0.124	23.879±0.065	23.818±0.044	0.5218	P>0.05, ns	P>0.05, ns	P>0.05, ns
Cooking loss(%)	25.653±1.760	26.660±0.357	27.495±0.213	0.1931	P>0.05, ns	P>0.05, ns	P>0.05, ns
Warner-Bratzler shear force (kg)	2.822±0.349	2.775±0.057	2.937±0.041	0.1446	P>0.05, ns	P>0.05, ns	P>0.05, ns
Post-mortem pH_{45min} B	6.240±0.017	6.294±0.026	6.308±0.020	0.8249	P>0.05, ns	P>0.05, ns	P>0.05, ns
Post-mortem pH_{45min} L	6.038±0.176	6.020±0.023	6.001±0.016	0.7750	P>0.05, ns	P>0.05, ns	P>0.05, ns

Table 1. Summary genotype analyses and oligonucleotides used for RT-PCR

Locus		*c.472C>G*
Oligo-nucleotides for genotyping	Allele-frequency	C: 0.162 G: 0.838
	Allele-specific Oligo 1	5‘-ACTTCGTCAGTAACGGACGGCGCAGGCTCTCTGGTCCG -3’
	Allele-specific Oligo 2	5‘-GAGTCGAGGTCATATCGTGGCGCAGGCTCTCTGGTCCC -3’
	Allele-specific Oligo 3	5‘GAGTACCTCTTCGAAAGGCTCATGCTGTAGAGCGTGAATCCCGGT CTGCCTATAGTGAGTC - -3’
Oligo-nucleotides for RT-PCR	F	5‘-ACCTACCTCTCTCTGGGCTT -3’
	R	5‘-GATGGTTTCTGCACGTCCTG -3’

Five milliliters of filtrate were diluted with 100 ml of deionized water. An aliquot (2 ml) of the diluted solution was placed into a test tube and mixed with 1 ml of oxidant solution [100 ml buffer solution (pH 6.0, 30 g citric acid + 15 g NaOH + 90 g sodium acetate trihydrate were dissolved in 500 ml deionized water before adding 290 ml 1-propanol) + 1.41 g chloramine-T]. The solution was mixed well and then held at room temperature for 20 min. One milliliter of color reagent was added to the treated solutions (10 g 4-dimethylaminobenzaldehyde dissolved in 35 ml perchloric acid (60% w/w), and then an added 65 ml of 2-propanol), heated in a water-bath at 60℃ for 15 min, and then cooled for 15 min. The absorbance was measured at 558 nm.

When significant differences were detected, the mean values were separated by the probability difference option.As a post hoc test, Mann-Whitney and Kruskal–Wallis test was employed to verify significant differences. Mann–Whitney and Student’s *t*-tests were used for the dominant and recessive models and ANOVA and the Kruskal–Wallis test were used for the codominant model. The analyzed group of dominant model was divided between wild type versus hetero + mutant type and that of recessive model was divided between mutant type versus hetero + wild type. The Mann–Whitney *U*-test was used for RT-qPCR result. The comparison was done by analysis mRNA expression of FTL in each tissue versus that in the lowest expressed tissue. $p < 0.01$ was considered to indicate

significance. Statistical analyses were double checked with Graph Pad prism (http://www.graphpad.com/scientific-software/prism/). All data are presented as means ± SEM.

RESULTS AND DISCUSSION

The expression pattern of *FTL* in various tissues of four pig breeds

To determine the expression patterns of *FTL* mRNA in various tissues, four different porcine breeds were examined. *FTL* mRNA was detected at high levels in the liver and kidney, the pivotal organs of defense and detoxification, in all breeds (Fig. 1).

The up-regulation of *FTL* may play an important role in these tissues. Consistent with our results, previous studies have shown that *FTL* mRNA is abundant in tissues that function in defense and detoxification, including the liver and kidney, in humans and mice (Su et al. 2004). With regard to *FTL* mRNA levels in the various organs, levels in the kidney and the liver were higher than other tissues. The mRNA expression of *FTL* in the kidney wasthe highest in all breeds, except Berkshire, where the highest levels were found in the liver.

Detection of synonymous SNPs in *FTL*

To identify SNPs in the *FTL* gene, RNA-seq was performed on the livers of Berkshire pigs. The *FTL* variant SNP c.472 C>G was detected.

Table 2. Analysis of the association between meat quality and FTL synonymous SNP*c.472C>G*

Model	Dominant			Recessive		
Genotype	CC (n=4)	CG+GG (n=412)	P value	GG (n=285)	CG+CC (n=131)	P value
Carcass weight (kg)	92.000 ± 1.732	85.765 ± 0.278	0.0212*	85.779 ± 0.350	85.924 ± 0.445	0.5649, ns
Meat color, CIE *a*	5.114 ± 0.414	6.150 ± 0.051	0.0472*	6.256 ± 0.063	5.858 ± 0.075	0.0060**
Meat color, CIE *b*	3.163 ± 0.379	2.872 ± 0.054	0.4801, ns	2.881 ± 0.067	2.862 ± 0.088	0.0003***
Drip loss (%)	4.368 ± 1.151	4.490 ± 0.091	0.7870, ns	4.676 ± 0.114	4.076 ± 0.144	0.0029**
Water holding capacity (%)	63.211 ± 1.174	58.206 ± 0.132	0.0034**	57.996 ± 0.153	58.813 ± 0.257	0.0092**
Post-mortem pH_{24hr}	6.036 ± 0.087	5.815 ± 0.010	0.0288*	5.812 ± 0.012	5.828 ± 0.019	0.5130, ns
Chemical composition, Collagen (%)	0.760 ± 0.005	0.889 ± 0.006	0.0488*	0.893 ± 0.007	0.878 ± 0.011	0.3148, ns
Chemical composition, Fat (%)	2.251 ± 0.324	2.801 ± 0.056	0.3322	2.813 ± 0.069	2.760 ± 0.094	p<0.0001***

Model	Codominant						
Genotype	CC (n=4)	CG (n=127)	GG (n=285)	P value	P value in CC vs. CG	P value in CC vs. GG	P value in CG vs. GG
CIE *a*	5.114 ± 0.414[a]	5.881 ± 0.075	6.256 ± 0.063	0.0005	P>0.05, ns	P>0.05, ns	P<0.01**
Drip loss (%)	4.36 ± 1.151	4.067 ± 0.144	4.676 ± 0.114	0.0116	P>0.05, ns	P>0.05, ns	P<0.01**
Water holding capacity (%)	63.211 ± 1.174	58.675 ± 0.253	57.996 ± 0.153	0.0012	P<0.05*	P<0.01**	P>0.05, ns

CIE *a* and*b* indicate meat color redness and yelowness.
The data are presented asmeans± SEM.
Significant differences between the genotype classes are indicated by ns (non-significance),* P <0.05, ** P < 0.01, and ***P < 0.001.

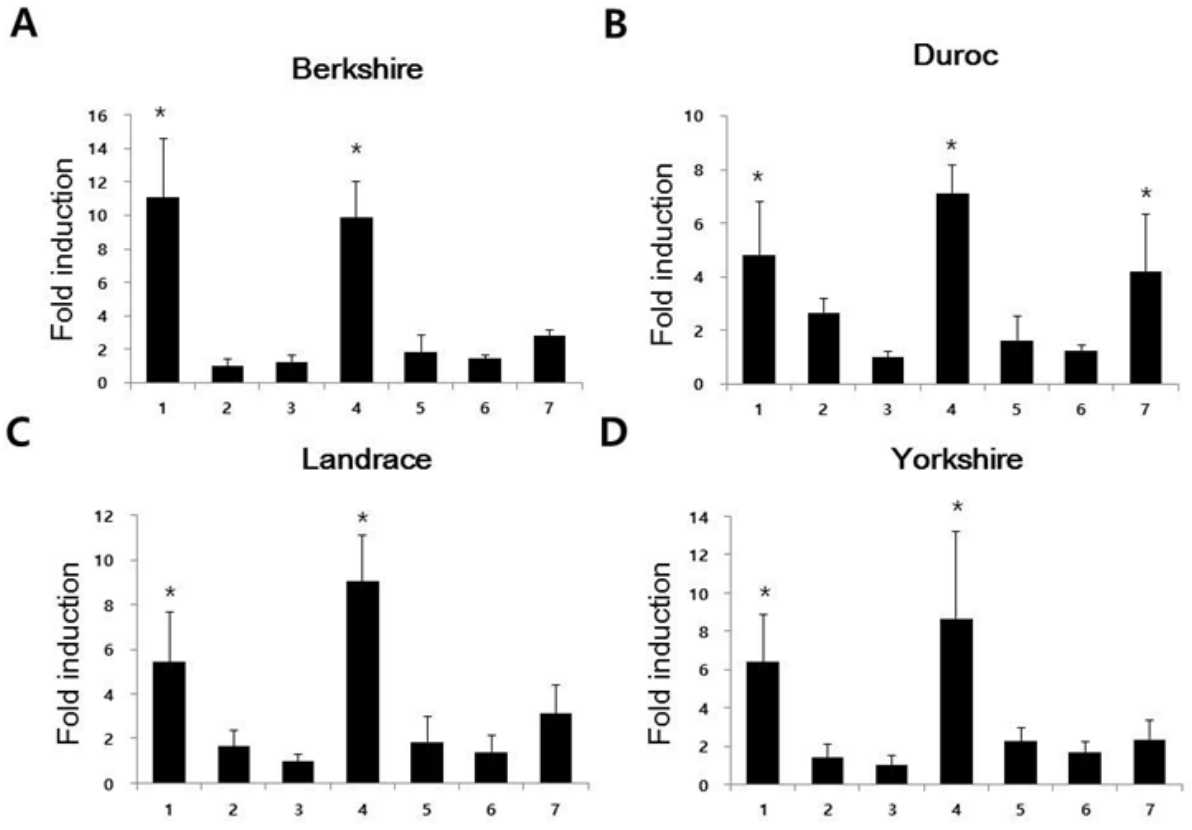

Figure 1.

The variant induced a change in the 3rd wobble base, but did not alter the amino acid (158 Pro), and was therefore a synonymous SNP. However, synonymous SNPs can still affect protein function. For example, a synonymous SNP in multidrug resistant gene 1 (MDR1) affects its translational and functional efficiency (Kimchi-Sarfaty et al. 2007). The oxidation of proteins is highly determinant of their physical and chemical properties, including solubility, conformation, and enzyme activities, and is an important factor for meat quality (Zhang, Xiao, and Ahn 2013). Lipid peroxidation is also a major factor in the deterioration of meat and meat products (Min and Ahn 2005). Since *FTL* plays a role in anti-oxidation and anti-lipid peroxidation, the up-regulation of *FTL* could be a crucial mechanism of protection against oxidation and lipid peroxidation (Harel and Kanner 1985; Boyer and McCleary 1987; Ahn, Wolfe, and Sim 1993; Ahn and Kim 1998). Together, these studies suggest that *FTL* may be a potential marker of meat quality. However, further studies are still needed to determine the underlying mechanism involved in *FTL* protection against oxidation and lipid peroxidation and how a synonymous SNP in *FTL* affects meat quality.

Evaluating the association between an *FTL* SNP and meat quality traits

To investigate the effect of the synonymous *FTL* SNP on meat quality, various tests for meat quality, including carcass weight, cooking loss, drip loss, water holding capacity, backfat thickness, meat color, chemical composition (protein, fat, collagen, and moisture), Warner-Bratzler shear force (kg), post-mortem pH was performed on 416 *longissimus dorsi* muscles (Table 2 and Table S1). In the dominant model, meat quality traits, such as carcass weight, meat color CIE *a*, water holding capacity, post-mortem $pH_{24hr,}$ and collagen contents, were significantly associated with *FTL* genotype, whereas in the recessive model, the meat color CIE *a,* CIE *b*, drip loss, and water holding capacity were notably correlated. In the codominant model, meat color CIE *a*, drip loss, and water holding capacity were significantly associated with *FTL* genotype (Table 2). CC homozygotes had noticeably higher carcass weight, water holding capacity, and post-mortem pH_{24hr} compared to GG homozygotes and CG heterozygotes in the dominant model, whereas GG homozygotes showed higher drip loss than the CC homozygotes and CG heterozygotes in the recessive model. These results indicate that the CC homozygotes have higher economic merit and meat quality than CG heterozygotes and GG homozygotes in Berkshire pigs. Therefore, *FTL* SNPs may be a useful factor in selecting pigs with higher meat quality. The *FTL* gene was expressed at the highest levels in the livers of Berkshire breed pigs. The *FTL* synonymous SNP *c.472 C>G* was significantly related to carcass weight, meat color (CIE *a*, CIE *b*), drip loss, water holding capacity, chemical composition (collagen, fat content), and post-mortem pH_{24hr}. In conclusion, a novel synonymous *FTL* SNP that is associated with meat quality traits was identified in Berkshire pigs. Therefore, we propose that *FTL* is an important gene associated with meat quality in Berkshire breed pigs. Our results provide useful information for the selection of economically attractive pigs.

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Supporting information

Table S1. Analyses of association between meat quality and FTL synonymous SNPc.472*C>G.*

Figure legends

Figure 1.Results of RT-qPCR in various organs and porcine breeds. (A) Berkshire, (B) Duroc, (C) Landrace, (D) Yorkshire. Total RNA was isolated from each organ and cDNA was synthesized using oligo-dT primers. The *FTL* mRNA expression levels were examined through RT-qPCR. The expression of *FTL* mRNA was normalized to a housekeeping gene. The relative expression amountsare presented for each pig breed. 1. Liver, 2. Stomach, 3.Lung, 4.Kidney, 5.Large intestine, 6.Small intestine, 7.spleen

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