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ORIGINAL ARTICLE

Association of a single nucleotide polymorphism in the 5′ upstream region of the porcine *myosin heavy chain 4* gene with meat quality traits in pigs

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ABSTRACT

We identified a potential molecular marker associated with meat quality traits in the *myosin heavy chain 4*, *MYH4* gene of Landrace pigs. Sequencing revealed a single nucleotide polymorphism (SNP; g.-1398G>T) in the 5′ upstream region of *MYH4*. It was significantly associated with the number of type IIa muscle fibers and water-holding capacity based on filter-paper fluid uptake. The GG genotype groups had a greater number of type IIa fibers and a larger area composed of type IIa fibers than the other genotype group ($P = 0.004$ and $P = 0.061$, respectively). Expression level of *MYH4* gene in the genotype TT or GT was higher than in genotype of GG ($P < 0.0001$). The T allele may enhance expression level of *MYH4* gene and then the portion of IIb type fiber in the muscle be increased by the T allelle. Therefore, we suggest that the g.-1398G>T in the 5′ upstream region of the porcine *MYH4* may be used as a molecular marker for meat quality traits, although its functional effect is not defined yet.

Key words: *meat quality, muscle fiber composition, myosin heavy chain 4 gene, pig, single nucleotide polymorphism.*

INTRODUCTION

Meat quality, one of the most important economic traits in farm animals, is controlled by multiple genes and is affected by many factors such as genetic effects of animal, muscle characteristics, production and environmental conditions. Understanding muscle formation and the metabolic pathways involved is important for meat production and quality in farm animals. One of the main factors determining muscle biochemical pathways is muscle fiber composition (Schiaffino & Reggiani 1996; Chang *et al.* 2003). The histochemical characteristics of muscle fibers are determined by the composition of myosin heavy chain (MyHC) isoforms (Kang *et al.* 2011). MyHCs are the major structural proteins of myosin filaments that, along with actin filaments, are able to convert chemical energy to mechanical energy for muscle contraction. MyHCs are encoded by a highly conserved multi-gene family and eight isoforms of them are known in mammals (Ia, IIa, IIx, IIb, embryonic, perinatal, slow/I/b and extraocular). Each isoform of MyHC encoded by a separate gene has its own adenosine triphospatase (ATPase) activity that is related to postmortem metabolism. There are four major fiber types in postnatal pig muscle characterized by the expression of the slow/I/b, IIa, IIx and IIb MyHC gene isoforms, encoded by *myosin, heavy chain 7, skeletal muscle* (*MYH7*), *MYH2*, *MYH1* and *MYH4* genes, respectively (Davoli *et al.* 2003). The slow/I/b and IIb fibers, also known as slow-oxidative and fast glycolytic fibers represent two extreme metabolic profiles, respectively. The IIa and IIx fibers are intermediate fast oxidative glycolytic fibers (Greaser *et al.* 2001). Composition of the fiber types is mainly related to postmortem metabolic rate and meat quality traits among various muscle fiber characteristics (Ryu & Kim 2005). In addition, the contents of the myosin heavy chain isoform can also have a profound influence on postmortem changes and meat quality, related to protein denaturation (Depreux *et al.* 2002; Eggert *et al.* 2002; Bowker *et al.* 2004). Also, some studies have described correlations between muscle fiber types or MyHC isoforms and meat quality (Calkins *et al.* 1981; Maltin *et al.* 1998; Tanabe *et al.* 2001; Eggert *et al.*

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2002; Chang *et al*. 2003). MyHC-IIb expression may be influenced by muscle disease. For example, Duchenne muscular dystrophy reduces the expression of MyHC-IIb (Webster *et al*. 1988). Studies of the masticatory muscles of growing mice have shown that when there is a shift from sucking to mastication, the expression of MyHC-IIb increases in the tongue and masseter muscles (Gojo *et al*. 2002; Maejima *et al*. 2005). In pigs, *MYH4* along with *MYH1* and *MYH2* are located in SSC12. The structure of the *MYH4* was previously characterized based on its complementary DNA (cDNA) sequence (Davoli *et al*. 1998). Moreover, a number of polymorphisms in the coding sequence of the porcine *MYH4* have been identified (Chikuni *et al*. 2001; Davoli *et al*. 2003). Also, the porcine *MYH4* has been suggested as a candidate gene on SSC12 for intramuscular fat content (Luo *et al*. 2012). However, until now, structural variation in the 5′ regulatory region of the porcine *MYH4* has not been reported. In this study, the role of the porcine *MYH4* on SSC12 was examined for its influence on meat quality. The 5′ regulatory region of the porcine *MYH4* was sequenced to identify genetic diversity, and then an association study and expression analysis of each genotype were conducted.

MATERIALS AND METHODS

The study protocol and standard operating procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science (Suwon, Republic of Korea).

Animals and trait measurement

A total of 117 pigs from five different breeds (26 Landrace, 23 Large White, 23 Duroc, 24 Berkshire and 21 Korean native pig (KNP) animals) were used to detect polymorphisms within the 5′ regulatory region of the porcine *MYH4*. A total of 133 Landrace pigs (40 castrated males and 93 females) were used in the association study. These pigs were fed the same commercial diet at the same pig farm until the average body weight reached 110 kg. Then they were slaughtered following standard procedures under the supervision of a Korean grading service for animal products. The muscle fiber characteristics were measured as previously described (Kim *et al*. 2012) (Table S1). In brief, the Longissimus dorsi muscles were collected within 45 min postmortem from the eighth thoracic vertebra and frozen immediately in liquid nitrogen. For histochemical analysis of the muscle fibers, serial transverse sections (10 μm thick) were prepared using a cryostat instrument (CM1850; Leica, Wetzlar, Germany) at -20°C and stained using actomyosin ATPase after acid preincubation (Brooke & Kaiser 1970). The stained samples were examined using an optical microscope equipped with a charge-coupled device color camera (IK-642K; Toshiba, Japan) and an image analysis system (Image-Pro Plus; Media Cybernetics, USA). The methods used to assess meat quality were previously described (Joo *et al*. 1999; Kim *et al*. 2012). To measure filter paper fluid uptake (FFU) showing water-holding capacity (Kauffman *et al*. 1986), the filter paper (Whatman #2, 42.5 mm in diameter) was pre-weighed, placed on the surface of a sample to absorb the fluids (< 2 s), and then weighed again. FFU was expressed as milligrams of exudate absorbed into the filter paper.

Single nucleotide polymorphism (SNP) detection and genotyping

Genomic DNA was extracted from ethylenediamineteraacetic acid (EDTA)-treated blood samples using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Approximately 2 kb of the 5′ regulatory region of the porcine *MYH4* was amplified using primers designed based on the published sequence of the gene (Acc. No. NC_010454; Table 1). The 5′ regulatory region was amplified from 117 genomic DNA samples of five different pig breeds and sequenced to detect polymorphic sites. PCR was performed in a volume of 20 μL containing 10 pmol each primer, 0.25 mmol/L each deoxynucleotide triphosphate (dNTP), 2 μL 10× PCR buffer, 1.25 U DNA polymerase (Genet Bio, Chungnam, Korea), and 100 ng genomic DNA. The thermal cycling conditions included an initial denaturation for 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 62°C, and 1 min at 72°C, with a final 10-min extension at 72°C in a DNA Engine Tetrad® 2 Thermal Cycler (Bio-Rad, Hercules, CA, USA).

To detect differences in the nucleotide sequences, direct sequencing of the PCR products was performed using a Big Dye Terminator Cycle Sequencing Ready

Table 1 Nucleotide sequences of PCR primers used for real-time PCR and 5′ regulatory region sequencing

Primer name		Sequence (5′→3′)	Application
MYH4	F	GCAGCAGGAGATTTCTGACC	Real-time PCR
	R	CAAGTTGGATGCGAAGGATT	Real-time PCR
	P1F	TCACATCTCTCCTCCCACCT	Promoter sequencing
	P1R	CGGGAGTTCTTTAAGACTTAGCA	Promoter sequencing
	P2F	CAAGGCTCTCTGACCCACTC	Promoter sequencing
	P2R	ACCGCATAATGATGGAAGGA	Promoter sequencing
GAPDH	F	GCAAAGTGGACATTGTCGCCATCA	Real-time PCR
	R	TCCTGGAAGATGGTGATGGCCTTT	Real-time PCR

Reaction Kit V3.0 (Life Technologies Corp., Carlsbad, CA, USA) and an ABI PRISM® 3730 Genetic Analyzer (Life Technologies Corp.). The sequences were compared to find SNPs using the SeqMan program (DNASTAR Inc. Madison, WI, USA). There was only one SNP at position -1398 in the porcine *MYH4*. Genotyping was performed in 133 Landrace pigs as part of an association study using direct sequencing with primer set P2 (Table 1).

Statistical analysis

An association analysis was performed using SAS 9.13 software (SAS Institute Inc., Cary, NC, USA). The following formula was used in a generalized linear model (GLM) analysis: $yijk = \mu + Gi + Sj + Pk + eijk$, where *yijkl* is the observed value, μ is the general mean, *Gi* is the fixed effect of genotype i, *Sj* is the fixed effect of sex j, *Pk* is the fixed effect of the period of slaughter k, and *eijk* is the random error. The results were presented as least squares means for each group and standard errors (SEs) of the least squares means. Multiple pairwise comparison for each genotype was performed using Tukey SEM to compute Tukey's Wholly Significant Difference post Q4 hoc test in R package semTools' (R version 3.1.1, USA). Significant differences among genotypes were separated using the probability difference option. Due to the small percentage (≤20%) of type I and IIa fibers and the large percentage (≥80%) of type IIb fibers, the total number of fibers was calculated using a logarithmic transformation; muscle fiber compositions were estimated using an angular transformation. The GLM procedure was used to analyze the genotypic expression levels determined using real-time PCR. Genotype, sex and period of slaughter were included as fixed effects in the statistical model. Differences were considered significant at $P < 0.05$. All data are expressed as the mean ± SE.

Expression analysis of porcine *MYH4*

Samples for quantitative RT-PCR were composed of 73 pigs including eight, 35 and 30 with GG, GT and TT genotypes, respectively. Total RNA was extracted from freshly harvested Longissimus dorsi muscle tissues with TRIzol reagent (Life Technologies Corp. Carlsbad, CA, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 μg total RNA with SuperScript III Reverse Transcriptase (Life Technologies Corp. Carlsbad, CA, USA). Reverse transcription was carried out at 50°C for 60 min in a 20 μL reaction volume containing 4 μL 5× First-Strand Buffer, 1 μL 0.1 mol/L dithiothreitol enhanced chemiluminescence (DTT), 1 μL 10 mM dNTP, 1 μL Recombinant RNasin® Ribonuclease Inhibitor (Promega, Madison, WI, USA), 200 U SuperScript III Reverse Transcriptase, 0.25 μg of random primers and 1 μg porcine total RNA. The primers used for the quantitative PCR were designed using the published porcine *MYH4* gene information (NM_001123141; Table 1). To investigate the expression pattern on each genotype, we performed quantitative PCR using cDNA synthesized from the Longissimus dorsi muscle of pigs. The quantitative PCR was performed in an ABI 7500 Real Time PCR System (Applied Biosystems/Life Technologies, Carlsbad, CA, USA). The relative expression of the *MYH4* gene was analyzed according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) using the *GAPDH* gene for normalization.

RESULTS AND DISCUSSION

SNP identification and genotype frequencies

Approximately 2 kb upstream from exon 1 of the porcine *MYH4* that had been obtained from the GenBank sequence (Acc. No. NC_010454) was amplified by PCR and directly sequenced to identify genetic variation in pig samples from all the five breeds used in this study. In this sequence analysis, one novel SNP site was found at *g*.-1398G>T in the 5′ regulatory region of *MYH4* gene only in the Landrace breed and deposited in dbSNP of National Center for Biotechnology Information (ss974514577), but genotypes of the other four breeds were exclusively GG at this site. In the Landrace, we observed that frequencies of genotypes GG, GT and TT at *g*.-1398G>T in the porcine *MYH4* were 0.385 ($n = 10$), 0.423 ($n = 11$) and 0.192 ($n = 5$), respectively. Hence, the frequency of G allele (0.596) was slightly higher than that of T allele (0.404) in the Landrace pigs (data not shown).

Association study

We performed an association study of the porcine *MYH4* genotypes with muscle fiber characteristics, meat production and meat quality traits in Landrace pigs (Table 2). The *g*.-1398G>T SNP of the porcine *MYH4* was significantly associated with the number of type IIa fibers ($P = 0.004$) and FFU ($P = 0.047$). FFU and a larger number of type IIa fibers showed a negative effect on *g*.-1398G>T SNP. The genotypes GG and GT had a larger number of type IIa fibers than genotype TT ($P = 0.061$), while FFU exhibited greater values in samples with the genotype GG. However, a number of type IIa fibers and FFU traits were not correlated in this study ($r = 0.055684$, $P = 0.5764$, data not shown). Although correlation between a number of type IIa fibers and FFU traits was not significant in this study, effects between those two traits were consistent with previous research (Ryu & Kim 2005). Ryu and Kim (2005) reported that the number of type IIa fibers was negatively correlated with FFU. Type I and IIb fibers, also known as slow-oxidative and fast-glycolytic fibers, respectively, represent two extreme metabolic profiles. Type IIa fibers are intermediate between type I and IIb fibers with respect to energy metabolism (Klont *et al.* 1998). Because muscle fibers contain different myosin heavy chains, which are responsible for their different ATPase activities (Picard *et al.* 1999), it is possible that fiber composition is associated

Table 2 Effects of g.-1398G>T in 5′ regulatory region of *MYH4* gene on muscle fiber characteristics and meat quality traits in Landrace pigs

Traits	Genotype			
	GG (n = 48)	GT (n = 54)	TT (n = 31)	P-value
Muscle fiber characteristics				
Total fiber number ($\times 10^3$)	1202 (51.3)†	1161 (39.4)	1175 (66.8)	NS
Mean CSA of fibers (μm^2)	4443 (148.2)	4349 (103.4)	4274 (162.0)	NS
The density of total fibers ($/mm^2$)	233.7 (8.37)	234.3 (5.85)	239.63 (9.15)	NS
Fiber number composition (%)				
Type I	8.83 (0.92)	10.04 (0.64)	10.82 (1.00)	NS
Type IIa	13.66[a] (0.89)	11.28[b] (0.62)	8.71[c] (0.97)	0.004**
Type IIb	77.55 (1.38)	78.70 (0.96)	80.48 (1.50)	NS
Fiber area composition (%)				
Type I	5.81 (0.60)	6.77 (0.42)	7.05 (0.65)	NS
Type IIa	7.93[a] (0.65)	7.07[b] (0.45)	5.50[c] (0.70)	0.061
Type IIb	86.25 (0.98)	86.17 (0.67)	87.45 (1.06)	NS
Meat quality				
pH_{45min}	6.08 (0.06)	5.99 (0.04)	6.03 (0.06)	NS
L^*	48.51 (0.55)	48.86 (0.42)	49.27 (0.63)	NS
a^*	6.56 (0.22)	6.24 (0.17)	6.61 (0.25)	NS
b^*	3.00 (0.18)	2.88 (0.14)	3.20 (0.20)	NS
Drip loss (%)	5.99 (0.45)	5.15 (0.35)	5.20 (0.53)	NS
FFU (mg)	65.74[a] (4.60)	52.05[b] (3.56)	59.73[c] (5.32)	0.047*

n, number of pigs; CSA, cross-sectional area; FFU, filter-paper fluid uptake. †Values are expressed as least squares means and standard errors.
[a,b,c]Least square means with different superscripts in the same row differ. *$P < 0.05$; **$P < 0.01$

with postmortem changes in the conversion of muscle to meat and subsequently meat quality (Karlsson *et al*. 1999; Brocks *et al*. 2000). In general, glycolysis and the onset of rigor mortis are faster in white than in red muscles. Type IIa and IIb fibers mainly carry out the glycolytic pathway, and their metabolism contributes to a quick decline in pH. Therefore, variation in fiber type can explain part of the variation in some meat quality traits (Essen-Gustavsson *et al*. 1994). Recent studies have shown correlations between muscle fiber characteristics and meat quality traits in cattle (Ozawa *et al*. 2000; Hwang *et al*. 2010) and in pigs (Karlsson *et al*. 1999; Eggert *et al*. 2002; Ryu & Kim 2005). In cattle, opposite effects were observed according to muscle fiber type I and type II (a and b) for meat quality. Muscle fiber type I was positively correlated with fat content and meat color L value, whereas muscle fiber type IIa and IIb were negatively correlated with ultimate pH, fat content and meat color L value (Hwang *et al*. 2010), In recent research, the porcine *MYH4* was suggested a positional candidate gene on SSC12 for intramuscular fat content by genome-wide association study using Illumina PorcineSNP60K chip (Luo *et al*. 2012). Association of a SNP in 3′ untranslated region of *MYH4* gene with visible intramuscular fat was reported (Davoli *et al*. 2003).

The GG and GT genotype groups had a higher number of type IIa fibers and a larger area composed of type IIa fibers than the other genotype group. Also, the GG genotype was significantly associated with a high FFU (water-holding capacity, P = 0.047). These results showed that, among various muscle fiber characteristics, fiber type composition was mainly related to meat quality traits.

Therefore, pigs with the TT genotype may produce more desirable meat than pigs with the GG genotype. Our results suggest that the porcine *MYH4* SNP *g*.-1398G>T can be a useful genetic marker to predict meat quality traits in pigs.

Effect of the SNP on *MYH4* gene expression in skeletal muscle

To examine whether the region including the porcine *MYH4* SNP *g*.-1398G>T is related to transcription factor binding sites, we conducted quantitative reverse transcription PCR for gene expression comparison between genotypes at *g*.-1398. The level of mRNA expression of *MYH4* gene was significantly higher in Landrace pigs of TT (0.698 ± 0.074) or GT (0.642 ± 0.067) genotypes rather than in those of GG (0.321 ± 0.141) genotypes ($P < 0.0001$) (Fig. 1). However, we could not find any transcriptional regulatory domain at g.-1398G>T. It was speculated that the T and G alleles may affect differential expression of *MYH4* gene and the proportion of IIa and IIb fibers in muscles, because pigs of the G alleles were significantly associated with a greater number of type IIa fibers and a larger area composed of type IIa fibers. This should be revealed by further study for the transcriptional regulatory function of the G and T allele of porcine *MYH4* gene. In conclusion, the SNP

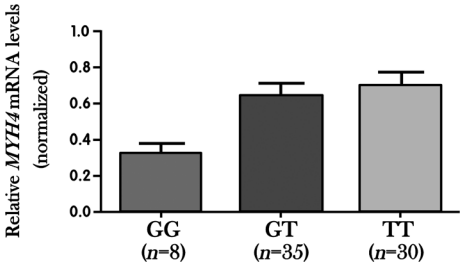

Figure 1 Comparison of messenger RNA (mRNA) expression levels of the porcine *MYH4* gene on g.-1398G>T site. Quantitative real-time PCR analysis was used to determine the expression levels of *MYH4* mRNA in the Longissimus dorsi muscle of each indicated genotyped Landrace pig. The mRNAs of *MYH4* were normalized to those of *GAPDH* and compared between different genotype. All values were described with mean ± standard error of the mean from three independent experiments. Different superscripts (a or b) above the error bar show significantly different genotypes on the single nucleotide polymorphism g.-1398 site ($P < 0.0001$).

g.-1398G>T may affect the mRNA expression of *MYH4*, muscle fiber composition, and subsequently meat quality in pigs. Therefore, we suggest that the SNP *g.*-1398G>T may affect the mRNA expression of *MYH4*, muscle fiber composition, and subsequently meat quality. However, further study is needed to confirm this result.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1 Means, standard deviations (SD) and ranges for measured traits.