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Association of functional sequence variants of the *MYH3* gene with muscle collagen contents in pigs

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Abstract

This study examined the association between functional sequence variants (FSVs) of *myosin heavy chain 3* (*MYH3*) genotypes and collagen content in a Landrace and Jeju native pig (JNP) crossbred population. Four muscles (*M. longissimus dorsi*, *M. semimembranosus*, *M. triceps brachii,* and *M. biceps femoris*) were used for the analysis of meat collagen content, and the same animals were genotyped for the FSVs of the *MYH3* gene by using PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism). Three FSVs of *MYH3* genotypes were identified and had genotype frequencies of 0.358, 0.551, and 0.091 for *QQ, Qq,* and *qq*, respectively. *QQ* animals for the FSVs of the *MYH3* genotypes showed higher collagen content in their *M. longissimus dorsi* ($p<0.001$), *M. semimembranosus* ($p<0.001$), *M. triceps brachii* ($p<0.001$), and *M. biceps femoris* ($p<0.001$) than *qq* homozygous animals. After the validation of this result in other independent populations, the FSVs of *MYH3* genotypes can be a valuable genetic marker for improving collagen content in porcine muscles and can also be applied to increase the amount of collagen for biomedical purposes.

1. Introduction

Native pig breeds are very important for the conservation and sustainable improvement of valuable economic traits in the future [1]. Native pigs in Korea, especially Jeju native pigs (JNP), have been extensively studied for crossbreeding with commercial pig breeds such as Landrace and Duroc and for quantitative trait locus (QTL) and genome-wide association study (GWAS) for identifying genetic markers of economic traits. The coat color of JNP is black, and the feed efficiency and growth rate of JNP are low. However, JNP has excellent meat quality characteristics, such as a solid fat structure, red meat color (a*), and high intramuscular fat (IMF) content, compared with commercial pig breeds, including Landrace [4].

Collagen is a major substrate protein in connective tissues such as skin, tendon, bone, and blood vessels in animals [6, 11]. Collagen is a lightweight protein that exists in epithelial cells and is widely distributed in multicellular animals such as invertebrates and vertebrates [13]. Collagen can form insoluble fibers with high tensile strength and has a triple hypochondrobar structure consisting of three identical polypeptide chains. Collagen is also the most abundant protein in vertebrates and makes up approximately 25% of the total protein in vertebrates [14]. Collagen comprises approximately 70% of the bones of organisms, approximately 50% of the cartilage connecting bones with bones in joints, approximately 70% of the dermis under the skin, and most vessels [20]. Collagen fibers exist in other forms between the perimysium and endomysium and are mainly composed of thick bundles of collagen in the perimysium and a fine network structure in the endomysium. Fibers depend on the type and area of collagen in the muscle, and collagen content's quantitative and chemical composition changes over time [12]. Collagen is classified according to its composition. Type I is the most abundant and strongest type of collagen found in the human body. It consists of eosinophilic fibers that form tendons, ligaments, organs, and skin. Type I collagen helps form bones and can be found in the gastrointestinal tract. In addition, collagen plays a major role in healing wounds, giving skin elasticity, and maintaining tissues. Type II collagen is mainly found in connective tissues and plays a role in forming cartilage. The health of the joints depends on cartilage composed of Type II collagen, which helps prevent various arthritis symptoms. Type III collagen is the main component of the reticular fiber and the extracellular matrix that makes up organs and skin. Collagen is found mainly in the Type I form and plays a role in skin elasticity and hardness. In addition, collagen forms blood vessels and tissues in the heart, so a deficiency in Type III collagen increases the risk of vascular rupture and premature death. Type IV collagen plays an important role in forming the basal lamina found in endothelial cells that form tissues surrounding organs, muscles, and fats, which cushion and protect tissues in the space between the top and bottom layers of the skin. The basal plate is necessary for the function of various nerves and blood vessels, so it constitutes most of the digestive organs and respiratory surfaces in the body. Type V collagen is needed to make up the cell surface as well as female placental tissue and hair strands [9, 10].

Collagen protein, a byproduct of meat production, is an important ingredient in food products such as casings. In addition, collagen is used in the production of cosmetics and is widely used as a healing aid following plastic surgery, bone reconstruction, and various dental and orthopedic surgeries. Collagen is used for cosmetic purposes to treat wrinkles and skin aging, as well as in vaccines and vitamins [15]. In addition, collagen has attracted attention from pharmaceutical and biomaterial-based packaging industries, as it can be used to encapsulate and form edible films [6]. Commercial collagen used for biomedical applications is extracted mainly from the skin of

76   pigs and cattle. However, the outbreak of prion diseases, such as bovine spongiform encephalopathy, has resulted

77   in anxiety among users of collagen derived from cattle. Interest in safer sources of collagen, such as pigs, has

78   greatly increased [16].

79   Recently, we reported evidence that a 6-bp deletion (XM_013981330.2: g.−1805_−1810del) in 5´-regulatory

80   region of *MYH3* is the first functional sequence variant for red meat color (a∗) and IMF in domestic pigs [4]. We

81   firstly mapped a 488.1-kb critical region in porcine chromosome 12 that influences both a^* and IMF by a combined

82   linkage-linkage disequilibrium analysis in two independent F_2 intercross between Korean native pigs (KNPs) and

83   Western commercial breeds (i.e., Landrace and Duroc). In this critical region, only the MYH3 gene, encoding

84   myosin heavy chain isoform 3, was found to be discriminatingly overexpressed in the skeletal muscle of KNPs

85   than in that of Landrace pigs. Subsequently, the MYH3 gene was verified as a causal gene for the two traits using

86   transgenic mice, and then detected the 6-bp deletion (XM_013981330.2: g.−1805_−1810del) variant in the 5´-

87   promoter region of the MYH3 gene for which Q allele carriers showed significantly higher values of a^* and IMF

88   than q allele carriers. Additionally, we demonstrated that this 6-bp deletion variant could abrogate the binding of

89   the regulatory myogenic regulatory factors (MRFs, i.e., MYF5, MYOD, MYOG and MRF4) and act as a

90   significantly weaker repressor, leading to increased expression of the MYH3 gene in the skeletal muscle.

91   Biological mechanism regarding myosin heavy chain and collagen has not well been known. Recently, Coelho *et*

92   *al*. reported that interaction of discoidin domain receptor 1 (DDR1) with myosin motors can contribute to collagen

93   remodeling [3]. However, biological relationship between the functional sequence variant (FSV) of MYH3

94   possessing the domain of myosin motors and collagen content needs to be further investigated. Therefore, the aim

95   of this study was to examine the association between FSVs located in the 5´-upstreme region of the MYH3 gene

96   and collagen content in crossbred JNP and Landrace pigs.

2.   Materials and Methods

2.1. Ethical approval

All experimental procedures were conducted according to national and institutional guidelines and approved by the Ethical Committee of the National Institute of Animal Science, Republic of Korea (Approval number: 2017-241).

2.2. Animals

A total of 187 animals of the F_4 resource population (103 male and 84 female) were established by crossing F_3 animals derived from intercrossing JNP and Landrace pigs. Animals were raised at the experimental farm of the National Institute of Animal Science, Jeju, Republic of Korea. They were fed *ad libitum*, and males were not castrated. All F_4 experimental animals were slaughtered in the same commercial slaughterhouse (Jeju Livestock Cooperative, Korea). The range of slaughter age of the pigs used for collagen content analysis was 180-200 days. The *M. longissimus dorsi*, *M. semimembranosus*, *M. triceps brachii* and *M. biceps femoris* from each carcass were used for the analysis of collagen content [19].

2.3. DNA extraction

Genomic DNA was isolated from blood and muscle using the sucrose-proteinase K method and used as a template for polymerase chain reaction (PCR) [18]. The absorbance of the separated DNA was measured using a NanoDrop ND-1000 spectrophotometer (USA), and then genomic DNA with an A260/A280 ratio of 1.8 or more was used as a template for PCR.

2.4. Collagen content

The collagen content was analyzed according to the method described by Choi *et al*. (2016)[5]. After 4 g of ground meat sample was placed into a triangular flask, 30 mL of a sulfuric acid solution was added. The sample was covered with a triangular flask in a dry oven for 16 hours before measurement. The hydrolyzable material was put in a 500 mL flask, diluted with distilled water, and filtered in a 100 mL triangular flask. 5 mL of the filtered solution was taken and diluted in 100 mL. Then, 2 mL of the final diluent was put in a 10 mL test tube, and 1 mL of the oxidizing solution was added. In the blank, 2 mL of distilled water and 1 mL of an oxidation solution were added instead of the diluent. samples were incubated at room temperature for 20 minutes, dissolved in 4-dimethyl-aminobenzaldehyde (60% w/w) in a color reagent (35 mL perchloric acid), mixed with 1 mL of 2-propanol 65 mL), covered with a cap, and immediately heated in a constant-temperature water bath (60 °C) for approximately 15 minutes. The amount of hydroxyproline was measured from the standard curve, and the collagen content (%) was calculated by multiplying the hydroxyproline content by a constant of 8.

2.5. PCR amplification and polymorphism determination

A set of primers was designed for the detection of the FSVs in the *MYH3* gene using available sequence information (XM_013981330.2). PCR was performed using a Maxime PCR Premix kit, and reactions included 20 μl of reaction mixture including 100 ng of DNA, 0.5 nmol of *MYH3* promoter-specific primers (MYH3_1_F,

132   5'-TGGTCTTTCCTAATTGGTGACAT-3', MYH3_1_R, 5'-AGTTTTGAGCAAGGCTTTTGTT-3') and

133   distilled water (iNtRON, Korea). PCR conditions were as follows: initial heating was at 95°C for 5 min, followed

134   by 35 cycles of 30 s for denaturation at 94 °C, 30 s for annealing at 65°C, and 30 s for an extension at 72°C,

135   followed by a final extension at 72°C for 10 min in a Nexus PCR machine (Eppendorf, Germany). And the

136   amplicons were digested with the restriction enzyme, *HpyCH4IV* (NEB, USA). The PCR products were separated

137   on 2.5% agarose gels (Lonza, Switzerland) and visualized by UV illumination with a BioFACT 100 bp plus DNA

138   ladder marker (BioFACT, Korea) [4].

139   *2.6. Allelic and genotypic frequencies*

140   The allele frequencies and heterozygosity (he), polymorphic information contents (PIC), and χ2 values (*P*-values)

141   for *MYH3* FSVs were calculated using the CERVUS 3.0.3 program [7].

142   *2.7. Association analysis*

143   Putative outliers were deleted based on the ascertainment of normality using a Ryan-Joiner (RJ) evaluation in the

144   MINITAB program (Minitab Inc., USA). An RJ score ≥ 0.99 was used to ascertain normality. The following

145   general linear model (GLM) was fitted to the phenotype data to estimate the effect of the FSVs of *MYH3* on

146   collagen content using MINITAB:

147     $Y_{ijk} = \mu + sex_i + genotype_j + b_1 X_{ijk} + e_{ijk}$

148   where, Y_{iik} is the observed phenotype, μ is the mean value, $genotype_i$ represents the FSVs in the *MYH3* gene (*Q/Q*,

149   *Q/q,* and *q/q*), X is the carcass weight as a covariate, b_1 is the regression coefficient and e_{ijk} is the random residual.

150   We used Tukey's multiple comparison method to separate means and set the significance level at *p*<0.05. The

151   percent phenotypic variance of a trait explained by the *MYH3* FSV was computed by [(VAR$_{reduced}$-

152   VAR$_{full}$)/VAR$_{reduced}$] × 100, where VAR$_{reduced}$ and VAR$_{full}$ are the variances of residuals of a GLM without and with

153   genotypes of the *MYH3* FSV in the models.

154

3.   Results & Discussion

3.1 Distribution of FSV of the *MYH3* gene in the Landrace and Jeju native pig crossbred population

The FSVs in the *MYH3* gene were genotyped using PCR-RFLP in the Landrace and JNP crossbred population. Determination of the genotype of the FSV of the MYH3 gene was conducted by PCR amplification and subsequent *HpyCH4IV* digestion. The *q/q* genotype represents the *MYH3* homozygous genotype originating from Landrace and Duroc pigs; the *Q/Q* genotype represents the *MYH3* homozygous genotype originating from JNPs. The results indicated that both the *Q* and *q* alleles were segregating, and all three genotypes (*QQ*, *Qq*, and *qq*) were identified for the FSVs of *MYH3* (Table 1, Table 2, Figure 2; *Q* and *q* are the Landrace and JNP variants, respectively).

These FSVs in the *MYH3* gene had allele frequencies of 0.634 and 0.366 for the *Q* and *q* alleles, respectively. This indicates a higher frequency of the *Q* allele than the *q* allele because the favorable *Q* allele was selected for in this population. The FSVs in the *MYH3* genotype had frequencies of 0.358, 0.551, and 0.091 for *QQ, Qq,* and *qq*, respectively, indicating more heterozygous animals than homozygous animals were observed in this population. However, the chi-square test results revealed that the FSVs were not in Hardy–Weinberg equilibrium ($p<0.05$), indicating that artificial selection was applied to the *QQ* genotype animals used in this study. These results confirm our expectations that the selection pressure was applied to obtain more favorable alleles in the population. In addition, the PIC (polymorphic information content) value was found to be 0.357, suggesting that this population showed intermediate polymorphism for these FSVs (Table 2). In a previous study to identify this causal variant, the *Q* allele is associated with higher redness value (a*), increased IMF contents, higher Type1 muscle fiber area and increased myoglobin content compared to the *q* allele in the Landrace x JNP crossbred pig population [4, 8]. Therefore, we can improve the meat quality of JNP x Landrace crossbred population via the marker-assisted selection of the FSV of *MYH3*.

3.2   Association analysis between FSVs of *MYH3* genotypes and collagen content

The *MYH3* gene encodes a portion of myosin, a contractile protein, which is especially fundamental to the proper functioning the Sarboomer of striated/skeletal muscle. *MYH3* is also recognized as an embryonic myosin heavy chain because it was mainly overexpressed in early mammalian development [2]

In this study, collagen content was measured in four muscles (*M. longissimus dorsi*, *M. semimembranosus*, *M. triceps brachii* and *M. biceps femoris*) in the Landrace and JNP crossbred population. Before the association test, a normality test for collagen content was performed using the Ryan-Joiner method [17]. The results showed an RJ score of 0.990 or higher, indicating that the collagen content data followed a normal distribution and could be further analyzed (Figure 1).

Through analysis of the association between collagen content and FSVs of the *MYH3* gene, we found that individuals with the *QQ* genotype had higher collagen content than those with the *qq* genotype in *M. longissimus dorsi*, *M. semimembranosus*, *M. triceps brachii* and *M. biceps femoris* muscles (Table 3, $p<0.05$, $p=2.00\times10^{-16}$,

191    $p=6.66\times10^{-16}$ and $p=3.31\times10^{-5}$, respectively).

192   Pork collagen has been used since the beginning of artificial skin research, primarily to assist in the treatment of
193   burns and trauma. When a wound occurs on the skin, a temporary protective film such as a silicon dressing is
194   attached until the damaged skin recovers. This prevents water leakage from the body, absorbs exudate (liquid
195   coming out of the blood when there is inflammation), and prevents invasion and infection by bacteria from the
196   outside. Except for the application of a gel, the dressing is a porous membrane made using a polyurethane
197   membrane or chitin, a freeze-dried product of pig leather, or the like [16]. However, it is applied in limited
198   situations and is expensive. When we computed the percent variance of the *MYH3* FSV as the effect size, we
199   found that the *MYH3* FSV explained up to 39.7% of phenotypic variance (Table 3). This amount of effect size of
200   *MYH3* FSV can be regarded as a good indication that this FSV could be used in the MAS for improving collagen
201   content in this JNP x Landrace crossbred population. With the fixation of *QQ* animals, the collagen content in
202   pork can be improved, meaning that consumers can have better quality pork and that the pork industry can benefit
203   from increasing the value of nonpreferred pork collagen, which can be used for medical purposes. However, it is
204   necessary to conduct additional experiments in other independent populations to confirm the effect size of *MYH3*
205   FSV before executing the MAS for collagen content. Selection for pigs with more collagen may allow this research
206   to be applied in the biomedical area and provide profits to farmers.

207

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275

Tables and Figures

276

277   Table 1. Descriptive statistics for FSVs of *MYH3* genotypes. Muscle collagen content was analyzed in
278   a crossbred pig population (Landrace×Jeju native pig)

|	n	Mean	SE	SD	Min	Med	Max
M. longissimus dorsi	187	1.1212	0.018	0.2465	0.6287	1.0956	1.9746
M. semimembranosus	187	1.303	0.0204	0.2795	0.7143	1.2614	2.4495
M. biceps femoris	187	1.4036	0.0172	0.2358	0.7557	1.3941	1.9506
M. triceps brachii	187	1.3258	0.014	0.1914	0.8317	1.3193	1.7922
Carcass weight	178	76.973	0.86	11.47	47.2	77.3	119.2

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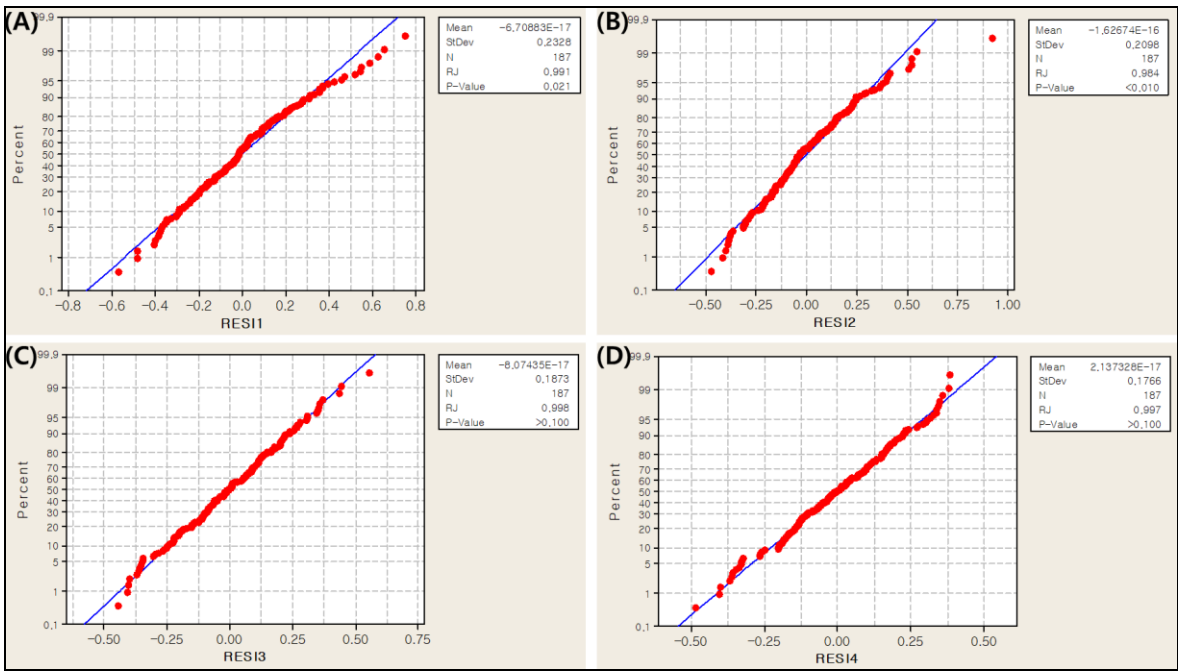
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283 Figure 1. Tests of normality by the Ryan-Joiner method. The vertical axis represents the scale of
284 probabilities, and the horizontal axis represents the scale of residual data. (A) *M. longissimus dorsi*; (B)
285 *M. semimembranosus*; (C) *M. triceps brachii* and (D) *M. biceps femoris dorsi*.

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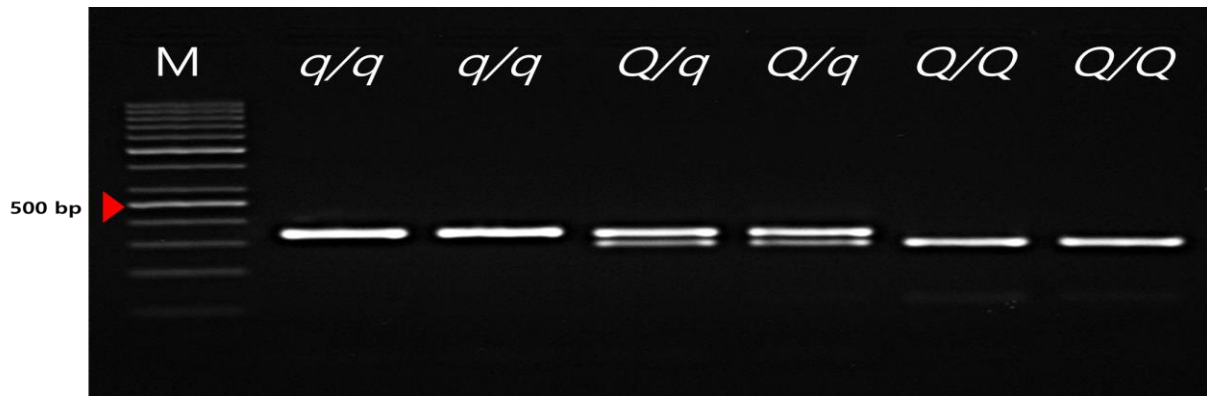
Figure 2. PCR amplification patterns for the polymorphisms of the FSVs in the porcine *MYH3* gene.
Allele *Q* and *q* showed on the agarose gel, respectively. M is 100-bp DNA ladder marker.

17

290 Table 2. Genotype frequency, allele frequency, and diversity parameters of the FSVs of the *MYH3*
291 variant in a crossbred pig population (Landrace×Jeju native pig)

FSVs of *MYH3* genotype frequency				Allele frequency		Diversity parameter*		
QQ (67)	*Qq* (103)	*qq* (17)	$\chi2^{\dagger}$	*Q*	*q*	H_o	H_e	PIC[1]
0.358	0.551	0.091	$5.726^{0.01}$	0.634	0.366	0.551	0.466	0.357

[*], H_o, H_e, and PIC indicate the values of observed heterozygosity, expected heterozygosity, and polymorphic information content, respectively.
[1] A PIC value < 0.25 indicates low polymorphism, $0.25 \leq$ PIC value ≤ 0.5 indicates intermediate polymorphism, and a PIC value > 0.5 indicates high polymorphism.
[†] Hardy-Weinberg equilibrium. Degree of freedom: 1

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293 Table 3. The association between FSVs of the *MYH3* genotypes and muscle collagen content in a
294 crossbred pig population (Landrace×Jeju native pig)

	Genotype†			%Var	*p*-value
	qq (17)	*Qq* (103)	*QQ* (67)		
M. longissimus dorsi	0.99 ± 0.06^a	$1.08 \pm 0.22^{a,c}$	$1.17 \pm 0.03^{b,c}$	4.9	0.015
M. semimembranosus	0.94 ± 0.05^a	1.22 ± 0.02^b	1.51 ± 0.03^c	39.7	2.00×10^{-16}
M. biceps femoris	1.09 ± 0.05^a	1.35 ± 0.02^b	1.54 ± 0.03^c	30.4	6.66×10^{-16}
M. triceps brachii	1.15 ± 0.04^a	1.30 ± 0.17^b	1.37 ± 0.02^c	10.3	3.31×10^{-5}

† Least Square Means (LSM) ± Standard Error

*Var is percentage of phenotypic residual variance explained by the *MYH3* FSV.

[a~c]Means with a different superscript in the same row significantly differ at *p*<0.05.

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