

Association of functional sequence variants of the *myosin heavy chain 3* gene with muscle collagen content in pigs

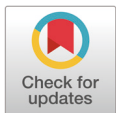
Yong-Jun Kang^{1#}, Sang-Hyun Han^{2#}, Sang-Geum Kim¹, Su-Yeon Kim¹, Hyeon-Ah Kim¹, Yoo-Kyung Kim¹, Ji-Hyun Yoo¹, Moon-Cheol Shin¹, Byoung-Chul Yang¹, Hee-Bok Park³, Jun Heon Lee^{4*} and In-Cheol Cho^{1*}

¹Subtropical Livestock Research Institute, National Institute of Animal Science, RDA, Jeju 63242, Korea

²Habitat Conservation Division, Korean National Park Research Institute, Korea National Park Service, Yongju 36015, Korea

³Department of Animal Resources Science, College of Industrial Sciences, Kongju National University, Yesan 32439, Korea

⁴Division of Animal and Dairy Science, Chungnam National University, Daejeon 34134, Korea



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#These authors contributed equally to this work.

*Corresponding author

Jun Heon Lee

Division of Animal and Dairy Science, Chungnam National University, Daejeon 34134, Korea.

Tel: +82-42-821-5779

E-mail: junheon@cnu.ac.kr

In-Cheol Cho

Subtropical Livestock Research Institute, National Institute of Animal Science, RDA, Jeju 63242, Korea.

Tel: +82-64-754-5716

E-mail: choic4753@korea.kr

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ORCID

Yong-Jun Kang

<http://orcid.org/0000-0001-8949-7831>

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 (*Musculus longissimus dorsi*, *Musculus semimembranosus*, *Musculus triceps brachii*, and *Musculus 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.

Keywords: Collagen content, Genotype, Muscle, pig, *Myosin heavy chain 3*

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

Sang-Hyun Han
<https://orcid.org/0000-0002-6615-2811>
Sang-Geum Kim
<https://orcid.org/0000-0002-0245-0465>
Su-Yeon Kim
<https://orcid.org/0000-0002-7989-7601>
Hyeon-Ah Kim
<https://orcid.org/0000-0002-4203-9857>
Yoo-Kyung Kim
<https://orcid.org/0000-0002-6685-7467>
Ji-Hyun Yoo
<https://orcid.org/0000-0003-1815-4115>
Moon-Cheol Shin
<https://orcid.org/0000-0001-6744-7155>
Byoung-Chul Yang
<https://orcid.org/0000-0002-2519-0352>
Hee-Bok Park
<https://orcid.org/0000-0002-9418-1898>
Jun Heon Lee
<https://orcid.org/0000-0003-3996-9209>
In-Cheol Cho
<https://orcid.org/0000-0003-3459-1999>

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

Conceptualization: Kang YJ, Han SH, Lee JH, Cho IC.
Data curation: Kang YJ, Han SH, Kim SG, Kim SY, Kim HA, Shin MC.
Formal analysis: Kang YJ, Kim YK.
Methodology: Kang YJ, Kim SG, Kim SY, Kim HA, Shin MC.
Software: Kang YJ, Kim YK.
Validation: Park HB, Lee JH, Cho IC.
Investigation: Kang YJ, Cho IC.
Writing - original draft: Kang YJ.
Writing - review & editing: Kang YJ, Han SH, Kim SG, Kim SY, Kim HA, Kim YK, Yoo JH, Shin MC, Yang BC, Park HB, Lee JH, Cho IC.

Ethics approval and consent to participate

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

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 [2].

Collagen is a major substrate protein in connective tissues such as skin, tendon, bone, and blood vessels in animals [3,4]. Collagen is a lightweight protein that exists in epithelial cells and is widely distributed in multicellular animals such as invertebrates and vertebrates [5]. 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 [6]. 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 [7]. 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 [8]. 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 [11]. In addition, collagen has attracted attention from pharmaceutical and biomaterial-based packaging industries, as it can be used to encapsulate and form edible films [3]. Commercial collagen used for biomedical applications is extracted mainly from the skin of pigs and cattle. However, the outbreak of prion diseases, such as bovine spongiform encephalopathy, has resulted in anxiety among users of collagen derived from cattle. Interest in safer sources of collagen, such as pigs, has greatly increased [12].

Recently, we reported evidence that a 6-bp deletion (XM_013981330.2: g.-1805_-1810del) in 5'-regulatory region of *myosin heavy chain 3* (*MYH3*) is the first functional sequence variant (FSV) for red meat color (a^*) and IMF in domestic pigs [2]. We firstly mapped a 488.1-kb critical region in porcine chromosome 12 that influences both a^* and IMF by a combined linkage-linkage disequilibrium analysis in two independent F_2 intercross between Korean native pigs (KNPs) and Western commercial breeds (i.e., Landrace and Duroc). In this critical region, only the *MYH3*

gene, encoding myosin heavy chain isoform 3, was found to be discriminatingly overexpressed in the skeletal muscle of KNP pigs than in that of Landrace pigs. Subsequently, the *MYH3* gene was verified as a causal gene for the two traits using transgenic mice, and then detected the 6-bp deletion (XM_013981330.2: g.-1805_-1810del) variant in the 5'-promoter region of the *MYH3* gene for which Q allele carriers showed significantly higher values of a^{*} and IMF than q allele carriers. Additionally, we demonstrated that this 6-bp deletion variant could abrogate the binding of the regulatory myogenic regulatory factors (MRFs, i.e., MYF5, MYOD, MYOG and MRF4) and act as a significantly weaker repressor, leading to increased expression of the *MYH3* gene in the skeletal muscle. Biological mechanism regarding myosin heavy chain and collagen has not well been known. Recently, Coelho *et al.* reported that interaction of discoidin domain receptor 1 (DDR1) with myosin motors can contribute to collagen remodeling [13]. However, biological relationship between the FSV of *MYH3* possessing the domain of myosin motors and collagen content needs to be further investigated. Therefore, the aim of this study was to examine the association between FSVs located in the 5'-upstream region of the *MYH3* gene and collagen content in crossbred JNP and Landrace pigs.

MATERIALS AND METHODS

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).

Animals

A total of 187 animals of the F₄ resource population (103 male and 84 female) were established by crossing F₃ animals derived from intercrossing JNP and Landrace pigs. Animals were raised at the experimental farm of the National Institute of Animal Science, Jeju, Korea. They were fed *ad libitum*, and males were not castrated. All F₄ experimental animals were slaughtered in the same commercial slaughterhouse (Jeju Livestock cooperatives, Jeju, Korea). The range of slaughter age of the pigs used for collagen content analysis was 180–200 days. The *Musculus longissimus dorsi*, *Musculus semimembranosus*, *Musculus triceps brachii* and *M. biceps femoris* from each carcass were used for the analysis of collagen content [14].

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) [15]. The absorbance of the separated DNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and then genomic DNA with an A260/A280 ratio of 1.8 or more was used as a template for PCR.

Collagen content

The collagen content was analyzed according to the method described by Choi *et al.* [16]. 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.

Polymerase chain reaction 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, 5'-TGGTCTTTCCTAATTGGTGACAT-3', MYH3_1_R, 5'-AGTTTTGAGCAAGGCTTTTGT-3') and distilled water (iNtRON, Seongnam, Korea). PCR conditions were as follows: initial heating was at 95 °C for 5 min, followed 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, followed by a final extension at 72 °C for 10 min in a Nexus PCR machine (Eppendorf, Hamburg, Germany). And the amplicons were digested with the restriction enzyme, *HpyCH4IV* (NEB, Ipswich, MA, USA). The PCR products were separated on 2.5% agarose gels (Lonza, Basel, Switzerland) and visualized by UV illumination with a BioFACT 100 bp plus DNA ladder marker (BioFACT, Daejeon, Korea) [2].

Allelic and genotypic frequencies

The allele frequencies and heterozygosity (he), polymorphic information contents (PIC), and χ^2 values (*p*-values) for *MYH3* FSVs were calculated using the CERVUS 3.0.3 program [17].

Association analysis

Putative outliers were deleted based on the ascertainment of normality using a Ryan-Joiner (RJ) evaluation in the MINITAB program (Minitab, State College, PA, USA). An RJ score ≥ 0.99 was used to ascertain normality. The following general linear model (GLM) was fitted to the phenotype data to estimate the effect of the FSVs of *MYH3* on collagen content using MINITAB:

$$Y_{ijk} = \mu + \text{sex}_i + \text{genotype}_j + b_1 X_{ijk} + e_{ijk}$$

where, Y_{ijk} is the observed phenotype, μ is the mean value, genotype_j represents the FSVs in the *MYH3* gene (*Q/Q*, *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. We used Tukey's multiple comparison method to separate means and set the significance level at $p < 0.05$. The percent phenotypic variance of a trait explained by the *MYH3* FSV was computed by $[(\text{VAR}_{\text{reduced}} - \text{VAR}_{\text{full}}) / \text{VAR}_{\text{reduced}}] \times 100$, where $\text{VAR}_{\text{reduced}}$ and VAR_{full} are the variances of residuals of a GLM without and with genotypes of the *MYH3* FSV in the models.

RESULTS AND DISCUSSION

Distribution of functional sequence variants of the *myosin heavy chain 3* gene in the Landrace and Jeju native pig crossbred population

The FSVs in the *MYH3* gene were genotyped using PCR-restriction fragment length

polymorphism (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 and 2, Fig.1; *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 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 × JNP crossbred pig population [2,18]. Therefore, we can improve the meat quality of JNP × Landrace crossbred population via the marker-assisted selection (MAS) of the FSV of *MYH3*.

Association analysis between functional sequence variants of myosin heavy chain 3 genotypes and collagen content

The *MYH3* gene encodes a portion of myosin, a contractile protein, which is especially fundamental to the proper functioning of the sarcolemma of striated/skeletal muscle. *MYH3* is also recognized as an embryonic myosin heavy chain because it was mainly overexpressed in early mammalian development [19].

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

	n	Mean	SE	SD	Minimum	Median	Maximum
<i>Musculus longissimus dorsi</i>	187	1.1212	0.018	0.2465	0.6287	1.0956	1.9746
<i>Musculus semimembranosus</i>	187	1.303	0.0204	0.2795	0.7143	1.2614	2.4495
<i>Musculus biceps femoris</i>	187	1.4036	0.0172	0.2358	0.7557	1.3941	1.9506
<i>Musculus triceps brachii</i>	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

FSVs, functional sequence variants; *MYH3*, myosin heavy chain 3.

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

FSVs of <i>MYH3</i> genotype frequency			χ^2 ¹⁾	Allele frequency		Diversity parameter		
QQ (67)	Qq (103)	qq (17)		Q	q	H _o	H _e	PIC ²⁾
0.358	0.551	0.091	5.7260.01	0.634	0.366	0.551	0.466	0.357

¹⁾Hardy-Weinberg equilibrium. Degree of freedom: 1.

²⁾A PIC value < 0.25 indicates low polymorphism, 0.25 ≤ PIC value ≤ 0.5 indicates intermediate polymorphism, and a PIC value > 0.5 indicates high polymorphism.

FSVs, functional sequence variants; *MYH3*, myosin heavy chain 3; H_o, observed heterozygosity; H_e, expected heterozygosity; PIC, polymorphic information content.

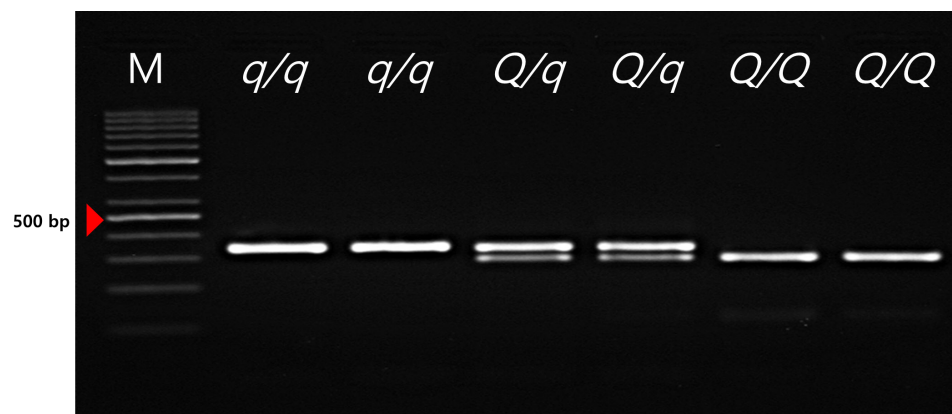


Fig. 1. PCR amplification patterns for the polymorphisms of the FSVs in the porcine *MYH3* gene. Allele Q and q showed on the agarose gel, respectively. M is 100-bp DNA ladder marker. PCR, polymerase chain reaction; FSVs, functional sequence variants; *MYH3*, myosin heavy chain 3.

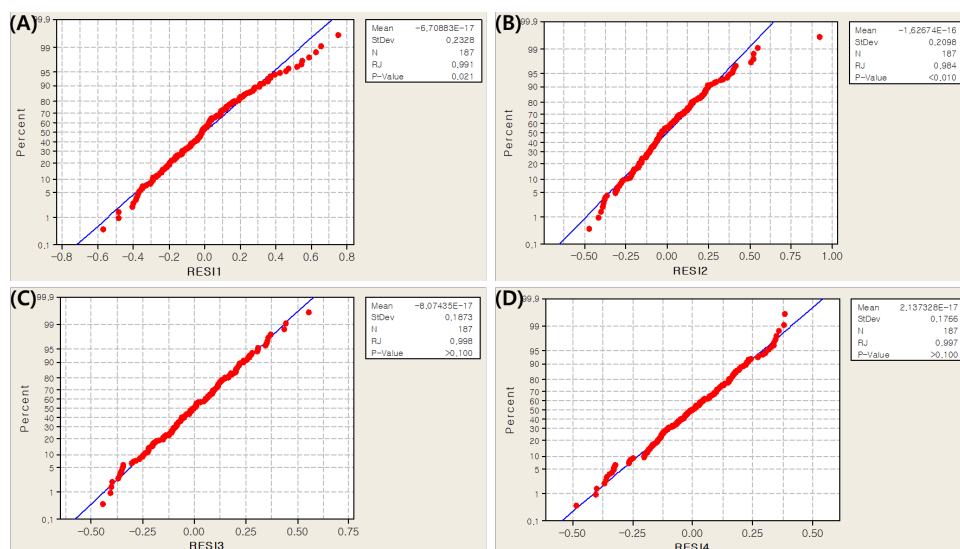


Fig. 2. Tests of normality by the Ryan-Joiner method. The vertical axis represents the scale of probabilities, and the horizontal axis represents the scale of residual data. (A) *Musculus longissimus dorsi*, (B) *Musculus semimembranosus*, (C) *Musculus triceps brachii* and (D) *Musculus biceps femoris dorsi*.

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 RJ method [20]. 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 (Fig. 2).

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, $p < 0.05$, $p = 2.00 \times 10^{-16}$, $p = 6.66 \times 10^{-16}$ and $p = 3.31 \times 10^{-5}$, respectively).

Pork collagen has been used since the beginning of artificial skin research, primarily to assist in

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

	Genotype ¹⁾			%Var ²⁾	p-value
	qq (17)	Qq (103)	QQ (67)		
<i>Musculus longissimus dorsi</i>	0.99 ± 0.06 ^a	1.08 ± 0.22 ^{bc}	1.17 ± 0.03 ^{bc}	4.9	0.015
<i>Musculus semimembranosus</i>	0.94 ± 0.05 ^a	1.22 ± 0.02 ^b	1.51 ± 0.03 ^c	39.7	2.00 × 10 ⁻¹⁶
<i>Musculus biceps femoris</i>	1.09 ± 0.05 ^a	1.35 ± 0.02 ^b	1.54 ± 0.03 ^c	30.4	6.66 × 10 ⁻¹⁶
<i>Musculus triceps brachii</i>	1.15 ± 0.04 ^a	1.30 ± 0.17 ^b	1.37 ± 0.02 ^c	10.3	3.31 × 10 ⁻⁵

¹⁾Least Square Means (LSM) ± SE.

²⁾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 $p < 0.05$.

FSVs, functional sequence variants; *MYH3*, myosin heavy chain 3.

the treatment of burns and trauma. When a wound occurs on the skin, a temporary protective film such as a silicon dressing is attached until the damaged skin recovers. This prevents water leakage from the body, absorbs exudate (liquid coming out of the blood when there is inflammation), and prevents invasion and infection by bacteria from the outside. Except for the application of a gel, the dressing is a porous membrane made using a polyurethane membrane or chitin, a freeze-dried product of pig leather, or the like [12]. However, it is applied in limited situations and is expensive. When we computed the percent variance of the *MYH3* FSV as the effect size, we found that the *MYH3* FSV explained up to 39.7% of phenotypic variance (Table 3). This amount of effect size of *MYH3* FSV can be regarded as a good indication that this FSV could be used in the MAS for improving collagen content in this JNP × Landrace crossbred population. With the fixation of *QQ* animals, the collagen content in pork can be improved, meaning that consumers can have better quality pork and that the pork industry can benefit from increasing the value of nonpreferred pork collagen, which can be used for medical purposes. However, it is necessary to conduct additional experiments in other independent populations to confirm the effect size of *MYH3* FSV before executing the MAS for collagen content. Selection for pigs with more collagen may allow this research to be applied in the biomedical area and provide profits to farmers.

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