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Association Study Between the Single Nucleotide Polymorphisms of *MUSTN1* Gene and Carcass Traits in Chickens

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ABSTRACT

MUSTN1 (Mustang, Musculoskeletal Temporally Activated Novel-1) gene is a musculoskeletal temporally activated novel gene which plays a key role in regulating the muscle development. In this study, the Single Nucleotide Polymorphisms (SNPs) in *MUSTN1* were scanned, the association of SNPs with carcass traits in chickens were analyzed by Polymerase Chain Reaction-single-strand Conformation Polymorphism (PCR-SSCP) and DNA sequencing methods. Two variations (2120T>C and g.2160A>G) were identified when screened its entire exons and partial introns in 638 chickens. The genotypes of g.2120T>C (Mutations in exon 2) were significantly associated with breast muscle weight, muscle fiber diameters ($p<0.05$). The genotypes of g.2160A>G (Mutations in exon 2) were significantly associated with breast muscle weight, breast muscle weight percentage ($p<0.05$) and great significantly associated with muscle fiber diameters ($p<0.01$). *MUSTN1* may act as a candidate gene of quantitative trait loci in regulating muscle growth.

Key words: Carcass traits, chicken, *MUSTN1,* PCR-SSCP, single nucleotide polymorphisms

INTRODUCTION

Muscle growth after birth increase of fibre sizes only (Moss and Leblond, 1971; Asakura *et al*., 2001; Hawke and Garry, 2001) because adult skeletal muscle fibers are terminally differentiated, its growth is resulted from increase of fiber sizes and regeneration by satellite cells (Hawke and Garry, 2001). A great number of studies have researched on MRFs family (MyoD, myogenin, myf-5 and MRF4) which participates in the process of myogenesis (Cornelison and Wold, 1997; Cooper *et al*., 1999; Cornelison *et al*., 2000; Asakura *et al*., 2001). *MUSTN1* silencing leads to downregulate of *MyoD* gene expression during myogenic differentiation (Liu *et al*., 2010) and reduce the expression of several myogenic marker genes (*MyoD*, *Myogenic*, *Myh4* and *Desmin*) (Gersch, 2010). Liu and Hadjiargyrou (2006) indicated that *MUSTN1* function could certainly place it within MyoD-associated signaling pathway. Thus *MUSTN1* gene may be linked to MyoD family which is candidates for causal roles in satellite cell activation.

MUSTN1 gene is a musculoskeletal temporally activated novel gene which plays a key role in regulating the muscle development (Lombardo *et al*., 2004; Zheng *et al*., 2009; Han *et al*., 2010) and chondrogenesis (Gersch and Hadjiargyrou, 2009; Gersch, 2010; Gersch *et al*., 2012). In addition, *MUSTN1* gene encodes an 82 amino acid nuclear protein which sharing high homology

with similar novel proteins in mouse, human and cow (Lombardo *et al*., 2004). In chicken, *MUSTN1* is located on chromosome 12 and consists of 3 exons and 2 introns, whose full-length DNA sequence is 3268 bp (as identified in NCBI databases: http://www.ncbi.nlm.nih.gov/). Previous work has identified *MUSTN1* as a small (9.6 kDa) protein that localizes predominantly to the musculoskeletal system in adult rodents (Livne *et al*., 1997).

During bone development and regeneration, *MUSTN1* expression was upregulated during muscle hypertrophy in sheep with the callipyge mutation (Vuocolo *et al*., 2007) and knockdown of *MUSTN1* could affect quantitative expression of the chondrogenic marker (Gersch *et al*., 2012). Liu *et al*. (2010) reported that after silencing, the *MUSTN1* did significantly inhibit myogenic fusion and differentiation. *MUSTN1* exists in embryos abundantly and skeletal muscle and tendon in adult tissues only, it plays a central role in the development and regeneration of the mammalian musculoskeletal system (Lombardo *et al*., 2004). In chicken, some reports have showed that *MUSTN1* plays an important role in skeletal muscle hypertrophy regulation (Zheng *et al*., 2009). However, by now, there is no report about the association study between the SNPs of *MUSTN1* and the economic traits in chicken. And in addition, our previous researches (Zhang *et al*., 2007, 2008, 2009; Wang *et al*., 2009; Zheng *et al*., 2009; Zhou *et al*., 2010; Xiao *et al*., 2011) have studied some candidate genes for finding the Quantitative Trait Loci (QTL) responsible for genetic variation and have found some genes such as *MEF2A, CAPN3, GH, MC3R, MC4R, ADP, CAPN1* and *CRBP2* are associated with the economic traits. It may offer a simple solution in improving the rate of genetic progress in economic traits with the development. The objective of this study is to investigate effects of genotypes on economic traits and provide useful information for selection in economic traits of chickens.

MATERIALS AND METHODS

Animal and tissue: In this research, we screened SNPs of the *MUSTN1* in 638 meat-type quality chicken populations including six commercial lines (S01, S02, S03, S05, S06 and D99) which developed in the form of pure-line selection by Sichuan Dahen Poultry Breeding Company using local breeds in Sichuan and Guangdong provinces of China (granted the pure-line certificate issued by Sichuan Province government) in 2008. Sixty to 150 chickens were randomly collected blood samples and slaughtered in each population and 638 chickens were used in total. Two pieces of the pectoralis and the crureus were cut with 2.0×0.5×0.5 cm from each carcass within 30 min postmortem and fixed in 4% polyphosphate formaldehyde. The muscle fiber diameter was measured from the fiber area. The muscle fiber density was calculated on video prints by a special morphometric facility, Motic mitic microscope. Chicken carcass traits were measured at 70 days of age at May, 2008. The estimated values of several important economic traits were detected the carcass traits: Live Weight (LW), Carcass Weight (CW), Eviscerated Weight (Ew), Semi-eviscerated Weight (SEW), Leg Muscle Weight (LMW), Breast Muscle Weight (BMW), Subcutaneous Fat Thickness (SFT), Abdominal Fat Weight (AFW), the Percentage of Breast Muscle Weight (BMWP) and the Percentage of Leg Muscle Weight (LMWP), density of muscle fiber (FD), Muscle Fiber Diameters (FDM). These carcass traits were described as previously (Zhang *et al*., 2009).

Procedure for PCR-SSCP and genotyping: PCR-SSCP method was used to analyze the *MUSTN1* polymorphisms. Five pairs of primers (Table 1) were designed from the reference sequences of *MUSTN1* in GenBank (Accession No. NC_006099.2) using Olige 6.0 and Primer Premier 5.0 softwares. This sequence is 3268 bp and CDS ranges from 76-84, 2131-2251, 2923-

Table 1: Primer pairs used in this study

Primer name	Primer sequences(5'-3')	Annealing temperature (°C)	Product length (bp)
E-1F	CACGCGGGCTGCATTTAA	61.4	217
E-1R	GCTTCACCGTAACCGAACAATC		
E-2AF	AGGGTGAGCAATCAGAAGACAAG	65.0	279
E-2AR	TCACTGGGTCTGGCTAATAAAGAG		
E-2BF	TAGCCAGACCCAGTGAAA	53.0	323
E-2BR	TCCAGACAGGGAGAATGA		
E-3AF	ATCCTTCACTGATTGCCTCCTC	64.5	245
E-3AR	TTGGGTATCTGTGGCTATGGTG		
E-3BF	ATTCCAGATTTCTACCCCTTGC	58.9	192
E-3BR	TGTCCAACAATCGTTTATTTACCA		

3029 bp, these five pairs of primers cover the whole exons and partial introns (5-221, 1865-2143, 2128-2450, 2882-3126 and 3075-3266 bp) of *MUSTN1*. Primers were synthesized by TaKaRa Biotechnology (Dalian, China). The PCR reactions were performed in a volume of 50 μL, including 25 μL 2×*Taq* PCR MasterMix including Mg^{2+}, dNTP and *Taq* DNA polymerase available (Beijing Tianwei Biology Technique Corporation), 2 μL each of forward and reverse primers (10 p mol μL^{-1}), 4 μL template (50 ng μL^{-1}) and 17 μL^{-1} ddH_2O. After 5 min of denaturing at 94°C, followed by 35 cycles at 94°C for 30 sec, annealed at different temperature (Table 1) for 30 sec, 72°C for 45 sec and a final elongation for 10 min. PCR products were resolved by electrophoresis on a 1.5% agarose gel and were detected on Gel DocTMEQ170-8060. 2.5 μL PCR products denaturated at 99°C for 11 min, then cooled on ice rapidly and loaded on 12% acrylamide/bisacrylamide (39:1) gels. After electrophoresis performing at 105 V for 10-13 h in 1×TBE buffer, gels were silver-stained. The PCR products of the different homozygous genotype were purified and sequenced by Invitrogen Biology Technique Corporation.

Statistical analyses: The carcass trait data was analyzed with SAS (SAS, 2004). Pairs of haplotype were build by the PHASE 2.0 program (Stephens *et al*., 2001). The SNP testing and the analyzed model were fixed effects:

$$Y = \mu+S+G+S\times G+e$$

Where:

Y = The trait measured the observation value
μ = The mean
S = The fixed effect of the strains
G = The fixed effect associated with the genotypes
S×G = The effect of interaction between the strains and the genotypes
e = The residual random effect

All data was described as least squares means±standard error. The significance of least square means was compared for significance using Duncan's test.

RESULTS

Sequence variations of the *MUSTN1*: Detecting the entire exon and partial intron of the *MUSTN1* on chicken carcass traits, g.2120T>C (Fig. 1) and g.2160A>G (Fig. not shown) were

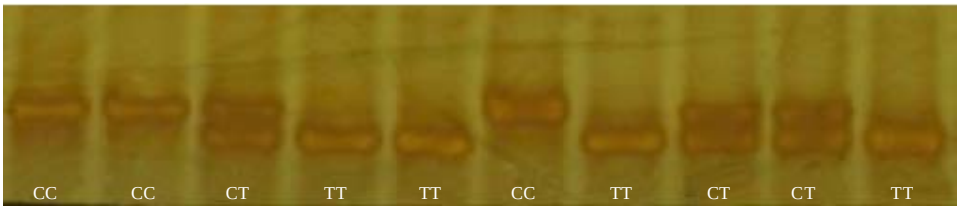

Fig. 1: Electropherograms of the polymorphic pattern for SNP g.2120T>C, the three genotypes of TT, CC and CT are marked in the figure, respectively

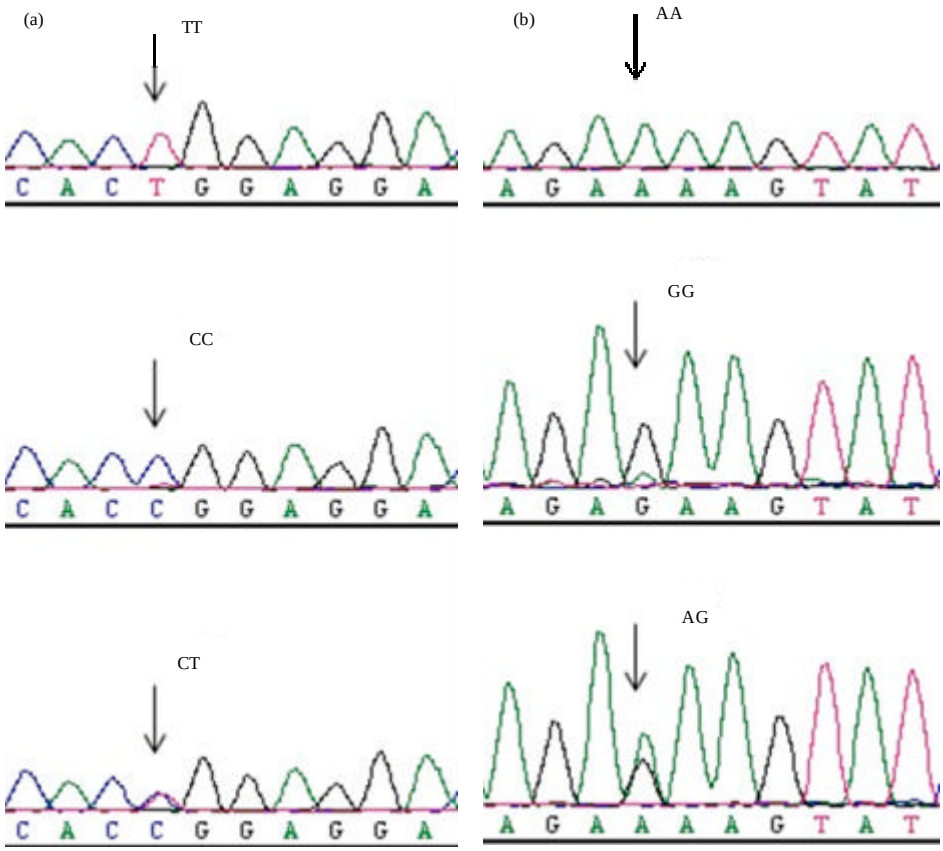

Fig. 2(a-b): (a) Sequence chromatogram of the polymorphic pattern for SNP g.2120T>C, the three genotypes of TT, CC and CT are marked in the chromatogram, respectively and (b) Sequence chromatogram of the polymorphic pattern for SNP g.2160 A>G, The three genotypes of AA, GG and AG are marked in the chromatogram, respectively

finally detected by directly sequencing the polymorphic fragment based on PCR-SSCP banding pattern in the second exon with the primers E-2AF/E-2AR and E-2BF/E-2BR, respectively (Fig. 2). Haplotype blocks were analyzed using the PHASE program to define the haplotype structures of the *MUSTN1*. A total of 8 SSCP profiles (H1H1, H1H2, H1H3, H1H4, H2H2, H3H3, H3H4 and H4H4) were detected, H1 (T-A), H2 (T-G), H3 (C-A) and H4 (C-G) were account for 72.96, 5.73,

Table 2: Haplotypes inferred based on the two single nncleotide polymorphisms of *MUSTN1*

	Sites		
Haplotypes	g.2120T>C	g.2160A>G	Frequency (%)
H1	T	A	72.96
H2	T	G	05.73
H3	C	A	09.18
H4	C	G	12.14

Table 3: Genotypes and allele frequencies of SNP1 in *MUSTN1* gene of 70 d in 638 meat-type chickens

					Genotype frequencies			Allele frequencies	
Loci	Position	Amino acid change	Lines	No.	TT	CT	CC	T	C
SNP1 g.2120T>C	Exon2	-	S01	140	0.743 (104)	0.100 (14)	0.157 (22)	0.793	0.207
			S02	120	0.667 (80)	0.167 (20)	0.167 (20)	0.750	0.250
			S03	116	0.810 (94)	0.034 (4)	0.155 (18)	0.828	0.172
			S05	124	0.806 (100)	0.097 (12)	0.097 (12)	0.855	0.145
			S06	070	0.686 (48)	0.200 (14)	0.114 (8)	0.786	0.214
			D99	066	0.515 (34)	0.273 (18)	0.212 (14)	0.652	0.348

Table 4: Genotypes and allele frequencies of SNP2 in *MUSTN1* gene of 70 d in 638 meat-type chickens

					Genotype frequencies			Allele frequencies	
Loci	Position	Amino acid change	Lines	No.	AA	AG	GG	A	G
SNP2 g.2160A>G	Exon2	-	S01	140	0.814 (114)	0.129 (18)	0.057 (8)	0.879	0.121
			S02	120	0.650 (78)	0.267 (32)	0.083 (10)	0.783	0.217
			S03	116	0.741 (86)	0.155 (18)	0.103 (12)	0.819	0.181
			S05	124	0.758 (94)	0.194 (24)	0.048 (6)	0.855	0.145
			S06	70	0.714 (50)	0.229 (16)	0.057 (4)	0.829	0.171
			D99	66	0.576 (38)	0.303 (20)	0.121(8)	0.727	0.273

9.18, 12.14% of the observations (Table 2), respectively. These variations were synonymous and did not change the amino acid sequence (Table 3, 4).

Association of the SNPs in *MUSTN1* with chicken carcass traits: The associated analysis of the *MUSTN1* SNPs with chicken carcass traits showed that two SNPs had significant effect on breast muscle fibre diameter and some carcass traits (Table 5). The genotypes of g.2120T>C were significantly associated with BMW, FDM ($p<0.05$). The genotypes of g.2160A>G were significantly associated with BMW, BMWP ($p<0.05$) and great significantly associated with FDM ($p<0.01$).

Association analyses showed that CT genotype of the chickens had significantly higher BMW, FDM than the CC or TT genotype ($p<0.05$); AG genotype of the chickens had significantly higher BMW, BMWP than the AA or GG genotype ($p<0.05$) and had significantly higher FDM than AA or GG genotype ($p<0.01$).

Haplotype construction and association with carcass traits: The diplotype H1H3 had a significantly advantageous effect on LMW, LMWP; H2H2 had a significantly advantageous effect

Table 5: Associatious between the *MUSTN1* gene SNPs and the chicken carcass traits

		Traits											
Loci	Genotypes	LW	CW	SEW	EW	BMW	LMW	BMWP	LWMP	AW	SFT	FDM	FD
SNP1	CC	1659.98±29.24	1478.59±27.27	1373.63±28.05	1148.85±21.99	177.87±4.62^{a}	241.64±5.71	15.46±0.25	20.77±0.25	40.32±2.55	0.40±0.01	30.44±0.67^{b}	629.33±31.03
	CT	1730.18±31.32	1540.76±29.21	1434.50±30.04	1199.93±23.55	193.25±4.95^{b}	259.22±6.11	16.12±0.26	21.35±0.27	33.04±2.74	0.37±0.01	32.40±0.67^{a}	650.38±30.42
	TT	1676.92±13.90	1492.30±13.14	1386.92±13.51	1162.89±10.59	180.89±2.23^{a}	248.95±2.75	15.56±0.12	21.19±0.12	35.80±1.23	0.37±0.01	30.38±0.36^{b}	667.47±16.50
	P	0.2212	0.2491	0.2828	0.2555	0.0481	0.1103	0.1196	0.2311	0.1315	0.1629	0.0291	0.5324
SNP2	AA	1670.30±13.81	1485.51±13.02	1380.26±13.39	1157.95±10.51	179.37±2.20^{a}	246.89±2.73	15.50±0.12^{a}	21.09±0.12	36.00±1.23	0.38±0.01	30.01±0.36^{A}	677.90±16.43
	AG	1724.72±24.78	1540.32±23.08	1435.17±23.73	1197.65±18.63	192.45±3.90^{b}	258.08±4.84	16.08±0.21^{b}	21.29±0.22	35.37±2.18	0.37±0.01	32.53±0.50^{B}	617.36±23.07
	GG	1669.81±40.88	1482.09±38.07	1373.98±39.15	1151.43±30.73	179.30±6.44^{a}	246.70±7.99	15.53±0.34^{a}	21.25±0.36	38.88±3.59	0.41±0.02	30.26±0.84^{A}	654.04±40.00
	P	0.1519	0.1088	0.1182	0.1585	0.0132	0.1252	0.0496	0.6804	0.6983	0.0878	0.0002	0.0904

The capital letters for the same line indicate that multiple comparison is greatly significant at $p<0.01$ and small letters indicate that multiple comparison is significant at $p<0.05$, LW: Live weight (g), CW: Carcass weight (g); SEW: Semi-eviscerated weight (g), EW: Eviscerated weight (g), BMW: Breast muscle weight (g), LMW: Leg muscle weight, BMWP and LMWP refer to the percentages of traits BMW, LMW relative to CW, respectively, AW: Abdominal fat weight (g), SFT: Subcutaneous fat thickness (mm), FDM: Breast muscle fiber diameters (μm), FD: Breast density of muscle fiber (fibers mm^{-2})

Table 6: Associations between the *MUSTN1* gene diplotypes and the chicken carcass traits

	Trsaits											
Diplotypes	LW	CW*	SEW*	EW*	BMW	LMW**	BMWP	LMWP*	AW**	SFT	FDM*	FD
H1H1	1675.69±14.89	1490.56±14.07	1384.69±14.48	1162.03±11.35	180.06±2.38	248.47±2.94	15.51±0.13	21.17±0.13	35.86±1.33	0.37±0.01	**30.00±0.39**	680.71±17.95
H1H2	1709.74±41.40	1528.15±38.59	1427.24±39.71	1190.83±31.12	191.56±6.53	257.28±8.05	16.09±0.35	21.33±0.36	35.20±3.63	**0.36±0.02**	32.61±0.84	569.56±38.30
H1H3	1744.63±100.39	1537.36±93.56	1442.19±96.28	1218.05±75.45	188.98±15.84	272.66±19.52	15.50±0.84	21.88±0.87	**23.67±8.80**	0.39±0.04	30.14±3.40	626.87±154.70
H1H4	1727.95±32.96	1540.48±30.71	1433.03±31.60	1197.41±24.77	193.57±5.20	257.63±6.41	16.18±0.28	21.29±0.29	34.05±2.89	0.36±0.01	32.45±0.67	653.00±30.63
H2H2	**1601.73±89.77**	**1419.57±83.67**	**1310.92±86.10**	**1091.40±67.48**	**167.85±14.17**	234.92±17.46	15.20±0.75	21.35±0.78	36.16±7.87	0.42±0.04	30.05±1.69	721.85±76.83
H3H3	1617.98±40.67	1441.15±37.90	1339.14±39.00	1119.39±30.56	172.82±6.42	**231.08±7.91**	15.40±0.34	**20.37±0.35**	39.51±3.56	0.39±0.02	30.13±0.97	641.71±44.06
H3H4	1784.20±100.34	1612.01±93.51	1502.91±96.23	1241.13±75.42	187.42±15.84	266.88±19.51	**15.06±0.84**	21.07±0.87	48.92±8.79	0.40±0.04	32.95±2.42	**561.43±110.09**
H4H4	1710.88±62.52	1527.71±58.84	1422.08±57.96	1187.42±46.11	184.29±8.53	257.71±12.49	15.49±0.40	21.60±0.55	35.69±4.44	0.40±0.03	31.33±0.73	639.95±22.77

Underline represents the advantageous diplotypes. Bold represents the negative diplotypes. All data are least squares means±standard error, *represents $p\leq0.05$, **represents $p\leq0.01$, LW: Live weight (g), CW: Carcass weight (g), SEW: Semi-eviscerated weight (g), EW : Eviscerated weight (g), BMW: Breast muscle weight (g), LMW: Leg muscle weight (g), BMWP and LMWP refer to the percentages of traits BMW and LMW relative to CW, respectively, AW: Abdominal fat weight (g), SFT: Aubcutaneous fat thickness (mm), FDM: Breast muscle fiber diameters (μm), FD: Breast density of muscle fiber (fibers mm^{-2})

on FD; H3H4 had a significantly advantageous effect on CW, SEW, EW, AW, FDM. While, diplotype H1H1 had a negative effect on FDM; H1H3 had a negative effect on AW; H2H2 had a negative effect on CW, SEW, EW; H3H3 had a negative effect on LMW, LMWP (Table 6).

DISCUSSION

Previously, Han *et al.* (2010) have researched the *MUSTN1* gene in porcine, they identified one single nucleotide polymorphism (c.265 C>T) by PCR-RFLP and found *MUSTN1* was significantly associated with weight, percentage of ham, proportion of lean and bone of the ham and meat color. In our study, two SNPs (g.2120T>C and g.2160A>G) had significant effect on breast muscle weight and muscle fibre diameter. According to our results, these variations were synonymous and did not change the amino acid sequence.

Haplotype diversity was always preferred in association analysis (Huang *et al.*, 2003). The genotypes of the two variations had the advantage heterozygous, TC and AG were both higher associated with BMW and FDM than the other homozygote. So, heterozygous had the advantage in *MUSTN1* of the chickens in this report. Diplotype H1H3 was found to be higher associated with LMW, LMWP than the other diplotypes, H2H3 was higher associated with FD than the other diplotypes and H3H4 was higher associated with CW, SEW, EW, AW than the other diplotypes. The genotypes of *MUSTN1* mainly affected the meat quality traits, especially in muscle growth.

A considerable amount of researches have been done about *MUSTN1* in muscle tissue during the last decade. Several studies revealed that *MUSTN1* gene plays an important role in bone and musculoskeletal system (Lombardo *et al.*, 2004; Liu and Hadjiargyrou, 2006; Gersch and Hadjiargyrou, 2009; Gersch, 2010; Han *et al.*, 2010; Liu *et al.*, 2010; Gersch *et al.*, 2012). Zheng *et al.* (2009) also reported *MUSTN1* could regulate the muscle hypertrophy between broilers and layers. But it is only a general analysis of the expression.

To the knowledge, this is the first systemic analysis of the association with the economic traits in *MUSTN1* in large-scale Chinese quality chickens. The data suggests that the *MUSTN1* may be closely linked with FDM from the association of the *MUSTN1* SNPs with chicken carcass traits and it may act as a candidate gene of quantitative trait loci in regulating muscle growth. But how it regulates in the muscle is unclear until now. Further studies are needed to clarify the various molecular mechanisms between the *MUSTN1* and muscle hypertrophy in chicken muscle.

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