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Original Article

The Single Nucleotide Polymorphisms of Myostatin Gene and Their Associations with Growth and Carcass Traits in Daheng Broiler

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ABSTRACT

Myostatin (*MSTN*) is a negative regulator of skeletal muscle growth. In order to investigate whether there is a correlation between *MSTN* polymorphisms and chicken production performance, in this study, single nucleotide polymorphisms (SNPs) in *MSTN* gene were examined across 180 Daheng broilers by direct sequencing of PCR product, and the correlations between the genotype and body weight at the age of 1-10 weeks and carcass traits at the age of 73 day were analyzed. Five SNPs (rs313622770, rs313744840, rs316247861, rs314431084, rs317126751) of *MSTN* gene were identified across *Daheng* broiler samples, and four haplotypes were reconstructed based on the five SNPs. Results of association analysis showed that four (rs313622770, rs313744840, rs316247861 and rs317126751) of these SNPs had significant association with some growth traits ($p<0.05$), but there were no significant effect on carcass traits and the four SNPs were strong linkage. For rs314431084, there was no significant correlation between different genotypes and growth or carcass traits. The AA genotype of rs313622770, GG genotype of rs313744840, CC genotype of rs316247861, TT genotype of rs317126751 were good for chicken growth. Diplotypes were significantly associated with chest muscle and leg muscle weight ($p<0.05$). Overall, these results provide evidence that polymorphisms in *MSTN* gene are associated with growth traits in chicken. The SNPs in *MSTN* gene could be utilized as potential markers for marker-assisted selection (MAS) during chicken breeding.

INTRODUCTION

Meat production is one of the most important economic traits in chicken, and how to improve meat production is one of the most important objectives of breeding researchers. The growth traits are regulated by multiple genetic loci. Recently, researchers have selected lots of candidate genes associated with growth traits, Myostatin (*MSTN*) is one of these genes identified as a negative regulation factor of skeletal muscle growth (Wehling *et al.,* 2000).

MSTN, also known as growth differentiation factor 8 (*GDF-8*), is a member of the transforming growth factor beta (*TGF*-β) family. It has been widely investigated in livestock, poultry, rodents and humans (Schiffer *et al.,* 2011; Varga *et al.,* 2003; Wang *et al.,* 2014). A number of evidence has shown that *MSTN* acts as a negative regulator of skeletal muscle growth, and loss or decrease of its activity will cause excessive development of animal muscle (Clop *et al.,* 2006). In the embryo stage, *MSTN* controls embryonic myoblast proliferation to regulate skeletal muscle size, Kocamis *et al. (*1999) investigated the developmental pattern of *MSTN* gene in chicken embryonic development and found that the expression of *MSTN* gene has been detected as early as the blastoderm stage, and they suggested *MSTN* gene

plays an important role in skeletal muscle development and embryogenesis in the chicken embryo. It also plays an important role in muscle regeneration and muscle wasting in adult animals (Sharma *et al.,* 2001). In adult mice, *MSTN* is mainly expressed in skeletal muscle. Some studies have found that the *MSTN* knockout mice is 30% heavier than wild-type mice, the skeletal muscle mass in *MSTN* knockout mice is 86% more than wild-type mice, and individual muscles of *MSTN* knockout mice weigh 2-3 times more than those of wild-type mice(Mcpherron *et al.,* 1997), it suggests that *MSTN* is the inhibitory factor of the skeletal muscle growth in adult mice. In addition, some reports also showed that *MSTN* regulates fat metabolism (Kim *et al.*, 2001; Lin *et al.,* 2002). Langley *et al.* (2002) have found that MSTN function is related to the *MyoD*, *MSTN* down-regulated *MyoD* to inhibit myoblast differentiation. In humans, SNPs of the *MSTN* gene are associated with obesity (Pan *et al.,* 2012) and gross muscle hypertrophy (Schuelke *et al.*, 2004). In livestock, the *MSTN* gene is widely studied for its association with muscular hypertrophy, some mutations of *MSTN* gene have been associated with double muscling in cattle (Gill *et al.*, 2009), and sheep (Dhakad *et al.,* 2017; Ranjan, 2017). Some SNPs were found in chicken *MSTN*. Zandi *et al.* (2013) found that *MSTN* had a high degree of polymorphism that significantly associated it with body weight in native chickens of Azerbaijan. Paswan *et al.* (2014) found a SNP in minimal promoter of *MSTN* that associated it with body weight in chicken. But there was little useful evidence of *MSTN* SNPs in chicken growth, it is necessary to study the relationship between SNPs of *MSTN* and chicken production traits.

Daheng broiler is a meat-type quality chicken population, it is a commercial broiler by a long-term breeding, and it is popularwith its excellent meat flavor in China. But its growth rate and meat production rate are much lower than those of international commercial broilers, such as Avain broilers. It is important to improve the growth traits of domestic commercial chicken. In this study, *MSTN* SNPs are identified to explore the relationship between their genotypes and growth, carcass traits in *Daheng* broiler, which provides the basic information for the marker-assisted selection in chicken.

MATERIALS AND METHODS

Experimental population

A total of 180 *Daheng* broiler from three strains were employed for testing, which were developed by Daheng Poultry Breeding Company (Chengdu, China), including S08 (30 females and 30 males), S07×S06 (30 females and 30 males) and S07×S08 (30 females and 30 males). All chickens were hatched on the same day and developed under the same conditions and diet. The BW (body weight) was measured in grams at hatch, 1wk (week), 2wk, 3wk, 4wk, 5wk, 6wk, 7wk, 8wk, 9wk and 10wk. All individuals were slaughtered at 73 days of age, after slaughtered, the carcass traits including live weight (LW), carcass weight (CW), eviscerated weight (EW), semi-eviscerated weight (SEW), breast muscle weight (BMW), and leg muscle weight (LMW) were measured and recorded on the same day. Before slaughtered, wing venous blood samples were collected, prepared for DNA extraction. Genomic DNA was isolated by the standard phenol/chloroform method, the purity and concentration of DNA samples were measured by Nucleic Acid Protein Analyzer Nanodrop 2000/2000C (Thermo, Germany). TE buffer was added to DNA samples extracted from the blood to produce a target concentration of 100ng/μL, then, the DNA samples were stored at -20°C.

All experimental procedures involving animals were approved by the Animal Care and Use Committee of College of Animal Science and Technology, Sichuan Agricultural University (No.YYS130125), and were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Amplification and genotyping

Primers for the chicken *MSTN* gene amplification and sequencing (Table 1) were designed in NCBI (National Center for Biotechnology Information) (Boschiero *et al.*, 2013) based on the complete DNA sequence of Gallus gallus *MSTN* gene (EMBL ID: ENSGALG00000039458).

Table 1 – Primer information for detecting SNPs in *MSTN* gene.

Primer name	Target region	Primer sequences (5'-3')	Annealing Temperature (°C)	Product(bp)
M1	Exon1	F: GGTTTTGACGACATGAGCCT R:ACGAAAGCAGCAGGGTTGTTA	52	540
M2	Exon2	F: TTTCTTTTTGTTCCCTGTTCAGT R: TCATCTGCCATTCTCGAAGCA	58.8	529
M3	Exon3	F: TCCCAGAAGGGTAGAAAGTTCAG R: TGTTGGCAATGCCTAGCGTA	52	648

Primer synthesis was completed by the Beijing TSINGKE Biological Technology Corporation (Beijing, China). PCR was carried out using a Gene Amp PCR System 9700 (Bio-Rad, USA) thermal cycler. The PCR reaction (25μL) contains 15μL 2×Taq MasterMix, 0.5μL forward primer (10nmol/L), 0.5μL reverse primer (10nmol/L), 1μL DNA, and 8μL ddH_2O. PCR cycles included 94°C for 2min; 35 cycles included 94°C for 45s, 52°C for 35s, and 72°C for 60s; and a final extension included 72°C for 10 min, ending with incubation at 4°C (Barnes, 1994). PCR products were sequenced by TSINGKE Biological Technology Corporation (Beijing, China).

Statistical analysis

The general linear model (GLM) procedure of SAS 6.12 (Statistical Analysis Systems Institute Inc. Cary, NC) was built to test associations between the genotype and growth traits, significant associations were declared when $p<0.05$, the mixed model is as follows:

$Y = \mu + G + S + B + F + e_{ijkf}$

Where Y = the dependent variable, μ= the population mean, G = genotype value, S = fixed effects of sex, B = fixed effects of breed, F = family effect, and e_{ijkf} = random error.

The identified SNPs in this *MSTN* gene were tested for Hardy-Weinberg equilibrium, when $p>0.05$ indicated the genetic balance of population gene (Wigginton *et al.,* 2005). The linkage disequilibria D' and r^2 value of the SNPs were estimated by Haploview (Barrett *et al.,* 2005). Significance of the least squares means was tested with the Duncan's Multiple Range test. The polymorphism information content (PIC) was established (PIC>0.5 is high polymorphism, 0.25<PIC<0.5 is intermediate polymorphism, and PIC<0.25 is low polymorphism)(Elston, 2005).

Haplotypes were constructed based on each SNP of *MSTN* in all experimental animals by use of the PHASE program v. 2.0. The function of this program is to reconstruct haplotypes from the population data. The genetic status of the subjects was expressed as the combination of two haplotypes. The SAS 6.12 (Statistical Analysis Systems Institute Inc. Cary, NC) was used to analyze the associations between the Haplotypes and growth traits. Significant associations were declared when $p<0.05$.

RESULTS

Sequence polymorphism in chicken MSTN gene

In this study, the exons sequence of the *MSTN* gene were examined, a total of five SNPs (Table 2) have been detected in *MSTN* exon1 of *Daheng* broiler. They were genotyped in *Daheng* broiler to evaluate their genetic association with chicken growth and carcass traits by direct sequencing of PCR product. For each SNP(SNP1-SNP5), three genotypes were found in the total population. The genotypes, allele frequencies and the genetic information of the 5 SNPs are showed in Table 3. PIC test results indicate that SNP1, SNP2, SNP3 and SNP5were intermediate polymorphism

Table 2 – Detailed information of SNP1-SNP5 in chicken *MSTN* gene.

Markers	Source[1]	Chr position	Variation	Function
SNP1	rs313622770	7/218133	G/A	cds-synon
SNP2	rs313744840	7/218142	A/G	cds-synon
SNP3	rs316247861	7/218277	C/G	cds-synon
SNP4	rs314431084	7/218316	G/A	cds-synon
SNP5	rs317126751	7/218406	C/T	cds-synon

[1]: SNP1-SNP5 was released by NCBI with accession number.Chr, chromosome. cds- synonomous.

Table 3 – Genotypes, allele frequencies and the genetic information of the 5 SNPs.

SNPs	Genotype frequency			Allele frequency		PIC	P
SNP1	AA	AG	GG	A	G		
	37.78%	46.67%	15.55%	61.11%	38.89%	0.3624	0.994
SNP2	AA	AG	GG	A	G		
	5.56%	41.11%	53.33%	26.11%	73.89%	0.3114	0.7831
SNP3	CC	CG	GG	C	G		
	51.11%	43.33%	5.56%	72.78%	27.22%	0.3177	0.5769
SNP4	AA	AG	GG	A	G		
	74.44%	24.44%	1.12%	86.67%	13.33%	0.2044	0.846
SNP5	CC	CT	TT	C	T		
	15.56%	47.78%	36.66%	39.44%	60.56%	0.3636	1

PIC means polymorphism information content; P is the results of C^2 test of Hardy-Weinberg equilibrium.

(0.5>PIC>0.25), which could be good genetic markers, only SNP4 was low polymorphism (PIC<0.25). And all SNPs were in accordance with Hardy-Weinberg equilibrium (p>0.05).

Association of 5 *MSTN* SNPs with chicken growth and carcass traits

The factor analysis results indicated that the SNP1 was significantly associated with BW (body weight) at hatch (p=0.033), 1wk (p=0.042) and 8wk (p=0.044) but was not associated with other growth traits. The SNP2 was significantly associated with BW at hatch (p=0.031) and1wk (p=0.036) of age but was not associated with other growth traits. The SNP3 was only significantly associated with BW at hatch (p=0.048). The SNP4 was not associated with any growth traits. And the SNP5 was significantly associated with BW at hatch (p=0.041), 1wk (p=0.037) and 8wk (p=0.048) of age (Table 4). In addition, the results showed that there were no significant association of each SNP with any carcass traits (p>0.05) (Table 5).

Meanwhile, SNP1 chickens with AA genotype had a higher BW athatch at 1wk and8wkthan those with genotypes AG and GG (p<0.05). In SNP2, chicken with the GG genotype had significant higher weight athatch and 1wk than those with AG and AA genotypes (p<0.05).In SNP3, the CC genotype had significant higher hatch weight than those with GG genotype (p<0.05), and there was no difference between chickens with CC and CG genotypes (p>0.05). And in SNP5, chicken at hatch, 1wk and 8 wk had-significantly higher weights with the TT genotype than those chickens with the CC and CT genotypes (p>0.05) (Table 6).

Table 4 – Association of *MSTN* polymorphisms with chicken growth traits.

SNPs	Body weight (p value)										
	Hatch(g)	1wk(g)	2wk(g)	3wk(g)	4wk(g)	5wk(g)	6wk(g)	7wk(g)	8wk(g)	9wk(g)	10wk(g)
SNP1	0.033*	0.042*	0.698	0.76	0.992	0.772	0.674	0.1	0.044*	0.376	0.721
SNP2	0.031*	0.036*	0.191	0.775	0.308	0.893	0.769	0.14	0.245	0.498	0.653
SNP3	0.048*	0.08	0.45	0.502	0.435	0.54	0.893	0.289	0.299	0.587	0.665
SNP4	0.91	0.262	0.115	0.784	0.086	0.136	0.578	0.941	0.382	0.925	0.594
SNP5	0.041*	0.037*	0.634	0.79	0.944	0.732	0.667	0.171	0.048*	0.358	0.653

*p≤0.05; wk means week of age.

Table 5 – Association of *MSTN* polymorphisms with chicken carcass traits.

SNP	Carcass traits (p value of significant test)					
	LW(g)	CW(g)	SEW(g)	EW(g)	LMW(g)	BMW(g)
SNP1	0.377	0.396	0.377	0.396	0.412	0.828
SNP2	0.788	0.795	0.808	0.833	0.72	0.691
SNP3	0.795	0.848	0.807	0.782	0.803	0.241
SNP4	0.634	0.659	0.667	0.8	0.601	0.776
SNP5	0.237	0.315	0.324	0.342	0.405	0.791

LW=live weight, CW=carcass weight, SEW=semi-eviscerated weight, EW=eviscerated weight, BMW=breast muscle weight, LMW=leg muscle weight.

Table 6 – Association analysis between the SNP genotypes and growth traits.

SNP	Growthtraits	Genotypes			p-value
SNP1		AA	AG	GG	
	Hatch	37.68±0.36^{a}	36.55±0.32^{b}	36.29±0.56^{b}	0.033
	1wk	110.44±1.77^{a}	105.83±1.59	102.86±2.76^{b}	0.042
	8wk	1651.18±37.51^{a}	1542.86±33.57^{b}	1504.29±58.46^{b}	0.044
SNP2		AA	AG	GG	
	Hatch	35.4±0.93^{b}	36.49±0.34^{b}	37.44±0.3^{a}	0.031
	1wk	100±4.6	104.86±1.69^{b}	109.58±1.49^{a}	0.036
SNP3		CC	CG	GG	
	Hatch	37.41±0.31^{a}	36.56±0.34	35.40±0.94^{b}	0.048
SNP5		CC	CT	TT	
	Hatch	36.29±0.56^{b}	36.58±0.32^{b}	37.67±0.36^{a}	0.041
	1wk	102.86±2.75^{b}	105.81±1.57^{b}	110.61±1.79^{a}	0.037
	8wk	1504.29±58.52^{b}	1545.12±33.39^{b}	1651.52±38.12^{a}	0.048

Results are expressed as mean ± standard errors. Different letters indicate significant differences (p<0.05);

$^{a, b}$ Means there is no common superscript differ significantly within a row (p<0.05).

Construction of haplotypes and their associations with chicken growth and carcass traits

Haplotypes were constructed based on the 5 SNPs by using the Haploview program, haplotypes inferred from genotype data showed that four haplotypes were found, including H1 ('AGCAT' of 57.9%), H2 ('GAGAC' of 25.4%), H3 ('GGCGC' of 12.8%), H4 ('AGGAT' of 1.7%), and others (frequencies lower than 1%). According to the genotypes of 180 *Daheng* broilers, a total of 7 diplotypes were studied associated with growth and carcass traits that the frequencies were higher than 1%, including H1H1 (33.33%), H1H2 (31.11%), H1H3 (14.44%), H1H4 (3.33%), H2H2 (5.56%), H2H3 (8.89%), H3H3 (1.11%), and others (2.22%).

The association analysis indicated that there were significant associations between diplotypes and carcass traits (Table7), but no significant results were obtained for growth traits. Diplotypes were significantly associated with LMW and BMW ($p<0.05$). The H1H4 diplotype had significantly higher LMW and BMW than other diplotypes ($p<0.05$).

Table 7 – Association between diplotypes and carcass traits.

Diplotypes	LW	CW	SEW	EW	LMW*	BMW*
H1H1	2118.33±58.25	1903.83±54.23	1800.33±54.07	1529.33±45.53	163.09±6.01^{b}	112.09±3.19^{b}
H1H2	2199.82±60.3	1971.43±56.14	1869.11±55.96	1591.07±47.13	169.09±6.22^{b}	118.49±3.3^{b}
H1H3	2274.17±92.11	2042.92±85.75	1931.67±85.49	1636.25±71.99	172.58±9.5^{b}	117.72±5.39^{b}
H1H4	2538.33±184.22	2305±171.5	2200±170.97	1881.67±143.99	231.48±19.01^{a}	149.45±10.08^{a}
H2H2	2104±142.7	1902±132.84	1792±132.43	1525±111.53	165.35±14.72^{b}	123.31±7.8^{b}
H2H3	2052.5±112.81	1847.5±105.02	1748.13±104.7	1485±88.17	154.54±11.64^{b}	110.29±6.17^{b}
H3H3	1920±319.07	1735±297.04	1625±296.13	1435±249.4	133.61±32.92^{b}	106.58±17.45^{b}
other	2215±225.62	1987.5±210.04	1865±209.4	1595±176.35	173.21±23.28	112.27±12.34^{b}

"*" means there is significant difference between Least mean squares for a certain trait; Bold represents the advantageous diplotype; Underline represents the negative diplotype^{a,b} means no common superscript differ significantly ($p<0.05$).

Linkage disequilibrium (LD) analysis

LD analysis was calculated from the genotypic data of 180 *Daheng* broilers. LD analysis (Fig.1A, B) indicates that SNP1, SNP2, SNP3 and SNP5 were strong LDs (D>0.8 and r^2>0.33), SNP4 and others were weak LDs (D<0.8 and r^2<0.33).

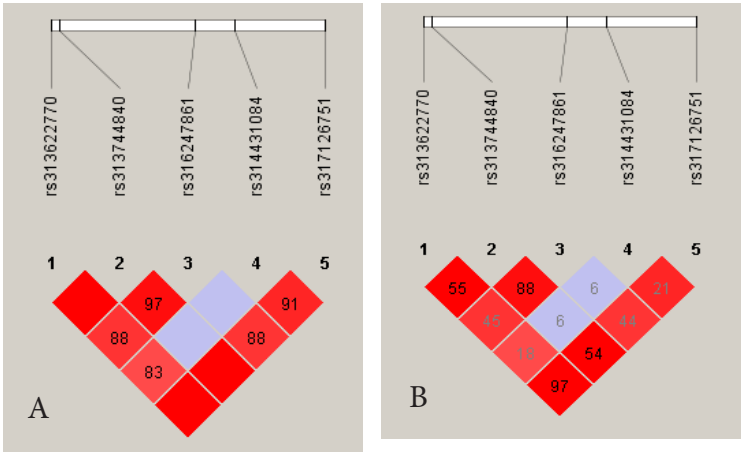

Figure 1 – Linkage disequilibrium (LD) of SNPs in the chicken MSTN exon1. (A) shows D', (B) shows r^2

DISCUSSION

Myostatin is a negative regulator of skeletal muscle growth, and loss of myostatin function will lead to a dramatic and specific increase in skeletal muscle mass (Lee & Mcpherron, 1999). The mutations that lead to loss of myostatin function have been found in these double-muscled cattle breeds, which is one of the reasons that myostatin accounts for double-muscling in cattle (Karim *et al.,* 2015). Therefore, it is important to investigate the associations and roles of *MSTN* SNPs in improving chicken growth performance.

A total of five SNPs have been detected in *MSTN* exon of *Daheng* broiler, all of them are associated with some growth traits, except SNP4, but there was no significant association of each SNP with any carcass traits, *MSTN* gene not only regulates muscle growth, but is also involved in fat metabolism. Lin *et al.* (2002) studied the muscle and fat growth in the myostatin knockout mice and found that myostatin knockout increased muscle growth, but decreased fat depots at 12 weeks, compared with wild type mice. Next, it is necessary to study the effect of *MSTN*SNPs in adipose tissue. Previous research also found that SNP2 was associated with body weight in chicken (Mitrofanova *et al.*, 2017). All the four SNPs (except SNP4) have significant effect on hatch. It has been reported that *MSTN* controlled embryonic myoblast proliferation to regulate skeletal muscle size (Dushyanth *et al.,* 2016). Zhang *et al.* (2012) found SNP4 was significantly associated with body weight in *Bian* chicken, the genotypes AA and GA had significantly higher body

weights than those of genotype GG, but in this study, there is no significant correlation between different genotypes and body weight in SNP4. It is likely to be caused by the lower genotype frequency of GG (1.12%) in *Daheng* broiler (Table 3) the commercial broiler compared with Bian chicken-the native breed. Commercial broiler is generated with a long-term breeding, the disadvantaged genotype was eliminated gradually during the breeding process.

Both SNP1 and SNP5 have a significant effect on body weight athatch, 1wk and 8wk. Linkage disequilibrium (LD) analysis indicate that SNP1 and SNP5 are a close LD pair (D'=1 and r^2=97) (Fig. 1A, B). Meanwhile, SNP1, SNP2, SNP3 and SNP4 have strong linkage (D>0.8 and r^2>0.33), which suggested that these four mutations are associated with some specific traits of interest, these results showed that the four SNPs have a significant effect on body weight at hatch which supports this conclusion. And the mutant AA genotype of SNP1 and mutant TT genotype of SNP5 are good for chicken growth, the mutant GG genotype of SNP2 and CC genotype of SNP3 are good for chicken early growth (Table 6). All of these SNPs are synonymous mutants, which were found to be significant associated with some growth traits in chicken, although synonymous SNPs do not cause any change in the amino acid and protein that they encode, they could affect mRNA stability, structure, splicing, or protein folding, which significantly affect protein function (Sauna, 2009).

It is reported that haplotype (diplotypes) determined the usefulness of closely link markers in identifying genetically superior individuals and was an essential part of the genetic architecture (Wang *et al.*, 2014). Kim *et al.* (2013) suggested that diplotypes were useful for identifying more precise and distinct signals over single-locus. Thus, it is necessary to analyze the effect of diplotypes on chicken growth and carcass traits and further find the application in marker-assisted selection. In this study, a total of 7 diplotypes were constructed to study their associations with growth and carcass traits, the results indicated that H1H4 diplotype had significantly higher LMW and BMW. There were some studies that revealed that MSTN haplogroups had a significant effect on body weight and carcass traits in chicken (Bhattacharya & Chatterjee, 2013; Dushyanth *et al.,* 2016). But in this study, H1H4 diplotype frequency is 3.33% within the limited sample population, we cannot conclude H1H4 was the most advantageous diplotype for chest muscle and leg muscle growth in *Daheng* broiler. It needs to be further verified.

In summary, five SNPs were identified in the chicken *MSTN* exon, four of those (SNP1, SNP2, SNP3 and SNP5) showed significant association with some growth traits in *Daheng* broiler. And they were strong linkage, except SNP4. Diplotypes were significantly associated with chest muscle and leg muscle weight, but the most advantageous diplotype needs to be further verified. Anyhow, *MSTN* SNPs could be the genetic markers for future MAS of chicken muscle development.

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