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, rs313744480, 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 mouse 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 has 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 popular with 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(℃) | Product(bp) |
|-------------|---------------|---------------------------|--------------------------|-------------|
| M1 | Exon1 | F: GGTITITGACGACATGAGCCT R: ACAGAAGCAGCGGGGTGTA | 52 | 540 |
| M2 | Exon2 | F: TTCTCTTTGTCTTGGCTGCTAGT R: TCATCTGCCATTCTCGAAGCA | 58.8 | 529 |
| M3 | Exon3 | F: TCCCAAAGGGGAGAGAAGTGCTAG R: TGTTGGCAATGCGCTAAGT | 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/μL), 0.5μL reverse primer (10nmol/μL), 1μL DNA, and 8μL ddH2O. 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:

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

Where $Y$ = the dependent variable, $\mu$= the population mean, $G$ = genotype value, $S$ = fixed effects of sex, $B$ = fixed effects of breed, $F$ = family effect, and $e_{ijk}$ = 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 SNP5 were intermediate polymorphism.

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

| Markers | Source | 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 37.78%          | AG 46.67%        | GG 15.55% | A 61.11% | G 38.89% | 0.3624 | 0.994 |
| SNP2 | AA 5.56%           | AG 41.11%        | GG 53.33% | A 26.11% | G 73.89% | 0.3114 | 0.7831 |
| SNP3 | CC 51.11%          | CG 43.33%        | GG 5.56% | C 72.78% | G 27.22% | 0.3177 | 0.5769 |
| SNP4 | AA 74.44%          | AG 24.44%        | GG 1.12% | A 86.67% | G 13.33% | 0.2044 | 0.846 |
| SNP5 | CC 15.56%          | CT 47.78%        | TT 36.66% | C 39.44% | T 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) and 1wk (p=0.036) of age but was not associated with other growth traits. The SNP3 was only SNP4 was low polymorphism (PIC<0.25). And all SNPs were in accordance with Hardy-Weinberg equilibrium (p>0.05).

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

| SNPs | 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  | 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   | 0.69   |
| 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  | Growth traits | Genotypes | p-value |
|------|---------------|-----------|---------|
| SNP1 | Hatch         | AA        | AG      | GG      | 0.033   |
|      |               | 37.68±0.36* | 36.55±0.32* | 36.29±0.56* |
|      | 1wk           | 110.44±1.77* | 105.83±1.59 | 102.86±2.76* | 0.042   |
|      | 8wk           | 1651.18±37.51* | 1542.86±33.57* | 1504.29±58.46* | 0.044   |
| SNP2 | Hatch         | AA        | AG      | GG      | 0.031   |
|      |               | 35.4±0.93*  | 36.49±0.34a | 37.44±0.3a  |
|      | 1wk           | 100±4.6    | 104.86±1.69b | 109.58±1.49b | 0.036   |
| SNP3 | Hatch         | CC        | CG      | GG      | 0.048   |
|      |               | 37.41±0.31a | 35.68±0.34 | 35.40±0.94a |
| SNP5 | Hatch         | CC        | CT      | TT      | 0.041   |
|      |               | 36.29±0.56a | 36.58±0.32a | 37.67±0.36a |
|      | 1wk           | 102.86±2.75a | 105.81±1.57b | 110.61±1.79a | 0.037   |
|      | 8wk           | 1504.29±58.52a | 1545.12±33.39b | 1651.52±38.12a | 0.048   |

Results are expressed as mean ± standard errors. Different letters indicate significant differences (p<0.05); * 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 Haplovew 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 (Table 7), 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±6.01b | 112.09±3.19b |
| H1H2       | 2199.82±60.3 | 1971.43±56.14 | 1869.11±55.96 | 1591.07±47.13 | 169.09±6.22 | 118.49±3.33 |
| H1H3       | 2274.17±92.11 | 2042.92±85.75 | 1931.67±85.49 | 1636.25±71.99 | 172.58±9.5 | 117.72±3.39 |
| H1H4       | 2538.33±184.22 | 2305±171.5 | 2200±170.97 | 1881.67±143.99 | 231.48±19.01 | 149.45±10.08 |
| H2H2       | 2104±142.7 | 1902±132.84 | 1792±132.43 | 1525±111.53 | 165.35±14.72 | 123.31±7.8 |
| H2H3       | 2052.5±112.81 | 1847.5±105.02 | 1748.13±104.7 | 1485±88.17 | 154.54±11.64 | 110.29±6.17 |
| H3H3       | 1920±319.07 | 1735±297.04 | 1625±296.13 | 1435±249.4 | 133.61±32.92 | 106.58±17.45 |
| other      | 2215±225.62 | 1987.5±210.04 | 1865±209.4 | 1595±176.35 | 173.21±23.28 | 112.27±12.34 |

**” means there is significant difference between Least mean squares for a certain trait; Bold represents the advantageous diplotype; Underline represents the negative diplotype

**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 r²>0.33), SNP4 and others were weak LDs (D<0.8 and r²<0.33).

**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 MSTNSNsPs 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 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).
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 at hatch, 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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