The single nucleotide polymorphisms of the chicken myostatin gene are associated with skeletal muscle and adipose growth

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Abstract  Myostatin, a new member of the TGF-β superfamily, is predominantly expressed in skeletal muscle cells and functions as a negative regulator of skeletal muscle growth in animals. Recently, we have reported three single nucleotide polymorphisms (SNPs) in the chicken myostatin gene. Herein, we investigate the association of those SNPs with the production traits in a F2 chicken line derived from Broilers crossing to Silky with the least square analysis. The results show that the BB and AA genotypes are strongly associated with abdominal fat weight (AFW), abdominal fat percentage (AFP), and birth weight (BW) ($P < 0.05$). Breast muscle percentage (BMP) of the AA type is higher than that of the AB type. The breast muscle weight and breast muscle percentages of F2 individuals have significant difference between CC and DD genotypes ($P < 0.05$). Breast muscle weight (BMW) of EF birds is higher than that of EE birds ($P < 0.05$). In this report, we present the first genetic evidence to show that chicken myostatin not only plays an important role in controlling skeletal muscle growth and differentiation, but also may be involved in regulation of adipose growth in chicken.

Keywords: myostatin gene, SNPs, F2 chicken population, abdominal fat, breast muscle weight.

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The transforming growth factor β superfamily is a large group of secreted factors that play indispensable roles in embryonic development and tissue homeostasis in adults. Myostatin, a new member of the TGFβ family, shares all the common features with other members of the TGF-β superfamily and is specifically expressed in skeletal muscle cells[1]. Myostatin protein is first synthesized in skeletal muscle as a 52 kDa propeptide and then prototypically processed at the conserved RSRR site to produce a 40 kDa latency-associated peptide and a 13 kDa mature peptide which then forms a homodimer (26 kDa) to bind to its receptor(s) for its biological function.

Gene knock-out experiments showed that myostatin null mice exhibited a two—threefold increase in the muscle mass compared to the normal wild type mice[1]. In addition, it was demonstrated that the loss of myostatin protein due to 11-bp deletion in the third exon or point mutations in coding region of myostatin...
gene could be a possible genetic cause for the double-muscle mass phenotype found in Belgian Blue and Piedmontese breeds of cattle\textsuperscript{[2–5]} These results provide strong evidence that myostatin is a negative regulator of skeletal muscle growth in animal\textsuperscript{[6,7]}.

Developmental studies of myostatin gene expression in mice have indicated that the myostatin gene is first expressed in myogenic precursor cell of the myotome compartment of developing somites\textsuperscript{[1]}, and later, in adult axial and paraxial muscles. The level of myostatin expression, however, varies in different axial and paraxial muscles. Recent studies have shown that myostatin is not only expressed in skeletal muscle, but also detected in some other tissues. Sharmam et al.\textsuperscript{[8]} report that myostatin is expressed in heart muscle of both fetus and adult mice. The myostatin mRNA is found in the porcine mammary gland. In addition, myostatin expression is not restricted to skeletal muscle in tilapia, as it occurs in many other tissues\textsuperscript{[9]}. Roberts et al. isolated two myostatin isoforms from the brook trout, one from muscle and brain, and the other from ovarian tissue\textsuperscript{[10]}. Although its functional roles in non-skeletal muscle are unknown, these results suggest that the biological function of myostatin is not limited to myogenesis, and it may affect organ formation and the development of other cell types.

The development of genetic markers for marker-assisted selection in animal agriculture has had a great impact on all animal genetics and breeding. Generally, in practice where a gene has been implicated in controlling quantitative traits (by chromosomal position relative to linkage peaks, known biological function, or expression pattern), it is desirable to exhaustively survey allelic variation for any association to the quantitative trait. Most sequence variations are attributable to SNPs, with the rest due to insertion or deletion of one or more bases, repeat length polymorphisms, and rearrangements. On average, SNPs occur every 1000—2000 bases and thus could be used as a genetic marker to systematically explore SNP variants for associations with quantitative traits. With the increase of SNP identification speed and efficiency, those studies will promote comprehensive tests of the hypothesis that common variants contribute significantly to the molecular nature of quantitative traits. Because of its functional role in controlling muscle mass, myostatin could be a potential candidate gene to be used as a molecular marker for molecular breeding program. Therefore, we have cloned the chicken myostatin gene and identified 5 SNPs of the chicken myostatin gene and revealed their genetic distribution in different chicken breeds\textsuperscript{[11]}, also analyzed effects of the SNPs on chicken growth in a F\textsubscript{2} resource population. Our results demonstrated that the SNPs are associated with abdominal fat weight (AFW), abdominal fat percentage (AFP), birth weight (BW) and breast muscle percentage (BMP). Notably, these data suggest that myostatin could be an ideal molecular marker for marker-assisted selection for skeletal muscle and adipose growth in chicken breeding program.

1 Materials and methods

1.1 Experimental chickens

The China Agricultural University (CAU) resource population was established by a reciprocal crossing of dwarf broilers and Chinese silky chicken. Three hundred and forty-five F\textsubscript{2} offspring from the population were used for this study.

The birds were slaughtered at 12 weeks of age, and the weight of carcass, breast muscle, leg muscle and abdominal fat were scored. Genomic DNA of birds was extracted from blood, and stored at \(-20\)°C for further use.

1.2 Primers

Three pairs of primers (P605\textsuperscript{5′}-TCCTATCAGGAATAACCTATC/P615\textsuperscript{3′}-ACCCTCAAGGAAATTCTGCAG, (P93\textsuperscript{5′}-CAACTTTTCAGTAATATGGAAATGAG, (P94\textsuperscript{3′}-TGATAGGTTTCTTGATAGGTA-3′) and (P80CTAACGTAGTAAACAAAGGCGACGCTAAACATTTATTCTAAATATTGATG-3′) were designed to detect the SNPs in chicken myostatin 5′- and 3′- regulatory region, respectively.

1.3 PCR condition

PCR amplification was performed in 25 µL in GeneAmp PCR System 9700 thermocycler (PE Ap-
plied Biosystem, USA). The reaction system comprised 50 ng genomic DNA, 10 mmol/L each dNTP, 0.2 µmol/L each primer, 1.5 mmol/L MgCl₂, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl and 1 U Taq. An initial 5 min denaturation at 94°C was followed by 35 cycles with the following conditions: 40 s at 94°C, 40 s at 56°C, 40 s at 72°C, and 10 min at 72°C.

1.4 SSCP analysis

PCR products were heated at 95°C for 8 min, then cooled on ice, and loaded onto the 12% acrylamide/bisacrylamide (29:1) gel with 5% glycerol. After running at 130 V for 10 h, the gel was stained using the silver staining method.

1.5 Cloning and sequencing

The PCR fragments from two homozygous genotypes were gel-purified, and ligated into T-Easy vector (Promage, USA). The recombinant plasmids were identified by EcoRI digestion and were sequenced to confirm the mutations.

1.6 Marker-trait association analysis

Estimate of the effect of the polymorphisms on growth traits was obtained by fitting a linear model: \( Y = \mu + \text{genotype} + \text{sex} + \text{reciprocal} + \text{family} + \text{hatch} + \text{residual} \). \( Y \) is carcass traits; \( \mu \) is the means of carcass traits. SAS software (version 6.12) was used for data analysis.

2 Results

2.1 Detection of genetic polymorphisms of chicken myostatin gene

The PCR products from 3 pairs of primers were analyzed by SSCP. Among 342 F₂ resource individuals, three genotypes were identified by primers p60/61 (fig. 1(a)), the homozygote type was defined as AA, and the heterozygote as AB. Three genotypes were identified with Primers p93/94 (fig. 2(a)), and two homozygote types were defined as EE and FF. Three genotypes were found with prime p80/81 (fig. 3(a)), with two kinds of homozygote defined as CC and DD respectively, and one kind of heterozygote named CD. The PCR products of homozygote AA, BB, EE, FF, CC and DD were cloned to T-Easy vector, and sequenced to detect the sequence difference. The sequence results show that one of the SNPs is localized in the 5′-regulatory region of the chicken myostatin gene due to 3 single point mutations (G304A, A322G, C334T, respectively) (fig. 1(b)), the other SNP in the 5′-regulatory region is a single point mutation of G167A (fig. 2(b)), and in addition, an A to T (7263) mutation is also identified in the 3′-regulatory region of this gene (fig. 3(b)).

2.2 Effect of myostatin SNPs on growth traits in chicken

The results of the least square analysis demonstrate that the birds with BB genotype have a signifi-

Fig. 1. SNPs analysis of chicken myostatin 5′-regulatory region (P60/61) (a) and sequence comparison between AA and BB genotypes (b).
Fig. 2. SNPs analysis in chicken myostatin 5'-regulatory region (P93/94) (a) and sequence comparison between EE and FF genotype (b).

Fig. 3. SNPs analysis in chicken myostatin 3'-regulatory region (P80/81) (a) and sequence comparison of CC and DD genotype (b).

cantly lower abdominal fat weight (AFW), abdominal fat percentage (AFP) and birth weight (BW) than birds with AA genotypes (table 1) ($P < 0.05$), while breast muscle percentage (BMP) of AA type is higher than that of AB type, the carcass and leg muscle weight have no significant difference among different genotypes AA, AB and BB. Breast muscle weight (BMW) of EF birds is higher than that of EE birds ($P < 0.05$) (table 2). Breast muscle weight of F$_2$ individuals is significantly different between CC and DD genotypes ($P < 0.05$). The breast muscle percentage is significantly different among the three genotypes (CC, CD and DD) ($P < 0.05$), with the mean of CC higher than that of CD and DD (table 3).

3 Discussion

Myostatin is autocrine signal protein secreted by muscle, and acts as a negative regulator of muscle growth. In addition, myostatin circulates in the blood of adult mice, and systemic overexpression of myostatin in adult mice is found to induce profound muscle and fat loss$^{[12]}$. Recent studies have revealed that myostatin-null mice have a significant reduction in fat accumulation with increasing age compared with wild-type littermates$^{[13]}$. It seems that the mice lacking myostatin gene might cause a switch between myogenesis and adipogenesis$^{[14]}$. Although the functional role of myostatin in controlling skeletal muscle growth has been well-documented, its physiological effect on adipogenesis remains unexplored. It is reported that myostatin inhibits preadipocyte differentiation in 3T3-L1 cells, which is mediated, in part, by altered regulation of C/EBP alpha and PPAR gamma$^{[15]}$. In this report, our results show that the birds with AA
Table 1  Effects of AA, AB and BB genotypes on abdominal fat weight (AFW), abdominal fat percentage (AFP), birth weight (BW), and breast muscle percentage (BMP)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Frequency</th>
<th>AFW</th>
<th>AFP</th>
<th>BW</th>
<th>BMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>116</td>
<td>0.340</td>
<td>52.55 ± 3.38a</td>
<td>0.0354 ± 0.0022a</td>
<td>30.79 ± 0.28a</td>
<td>0.0613 ± 0.001a</td>
</tr>
<tr>
<td>AB</td>
<td>187</td>
<td>0.548</td>
<td>49.10 ± 3.32ab</td>
<td>0.0353 ± 0.0022ab</td>
<td>30.35 ± 0.21ab</td>
<td>0.0592 ± 0.001b</td>
</tr>
<tr>
<td>BB</td>
<td>38</td>
<td>0.111</td>
<td>43.34 ± 5.02b</td>
<td>0.0294 ± 0.0038b</td>
<td>29.51 ± 0.45b</td>
<td>0.086 ± 0.0015ab</td>
</tr>
</tbody>
</table>

The difference is not significant for the means having the same letter. AFW, Abdominal fat weight; AFP, abdominal fat percentage; BW, birth weight; BMP, breast muscle percentage.

Table 2  Effects of EE, EF and FF genotypes on breast muscle percentage (BMP)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Frequency</th>
<th>BMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE</td>
<td>170</td>
<td>0.499</td>
<td>86.70 ± 3.32a</td>
</tr>
<tr>
<td>EF</td>
<td>131</td>
<td>0.384</td>
<td>93.23 ± 3.50a</td>
</tr>
<tr>
<td>FF</td>
<td>40</td>
<td>0.117</td>
<td>90.16 ± 4.56ab</td>
</tr>
</tbody>
</table>

The difference is not significant for the means having the same letter. BMP, Breast muscle percentage.

Table 3  Effects of CC, CD and DD genotypes on breast muscle weight (BMW) and breast muscle percentage (BMP)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Frequency</th>
<th>BMW</th>
<th>BMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>213</td>
<td>0.617</td>
<td>95.37 ± 1.57a</td>
<td>0.0634 ± 0.00058a</td>
</tr>
<tr>
<td>CD</td>
<td>126</td>
<td>0.365</td>
<td>89.93 ± 2.12ab</td>
<td>0.0614 ± 0.00078ab</td>
</tr>
<tr>
<td>DD</td>
<td>6</td>
<td>0.017</td>
<td>84.49 ± 7.29b</td>
<td>0.0544 ± 0.00269</td>
</tr>
</tbody>
</table>

The difference is not significant for the means having the same letter. BMW, Breast muscle weight; BMP, breast muscle percentage.

genotype have higher abdominal fat weight, abdominal fat percentage, birth weight, and breast muscle percentage than the birds with genotype BB. This is the first time that we provide genetic evidence to demonstrate an association between the SNPs in the chicken myostatin gene and adipose growth in chicken resource population.

During the myogenic process in vitro, proliferating myoblasts irreversibly withdraw from the cell cycle, acquire an apoptosis-resistant phenotype and fuse into mature myotubes. Transient expression of mouse myostatin in C2C12 myoblasts efficiently inhibits cell proliferation. When C2C12 myoblasts are incubated with recombinant myostatin, myoblasts proliferation is inhibited. It has been reported that myostatin prevents the progression of myoblasts from the G1 to S-phase of the cell cycle. However we know little about molecular mechanism of myostatin action as a negative regulator of myogenesis. p21, the cyclin-dependent kinase inhibitor, is the imperative factor regulating both myocyte cell cycle exit and viability during this transition. Biochemical studies indicate that there is an increase in p21 expression and a decrease in Cdk2 protein and activity in the myoblasts. In addition, the hypophosphorylated form of Rb protein is predominantly present in the myoblasts treated with myostatin protein. Therefore Thomas et al. propose a working model for myostatin function in controlling myoblast proliferation. Myostatin causes G1 growth arrest of myoblasts by the hypophosphorylation of Rb protein and inactivation of cycle E/Cdk2 by induced p21 [16–19].

Genetic marker is a commonly occurring genetic variation that can be easily tracked in genetic studies and can be used on entire alleles, repetitive stretches of DNA or single nucleotide polymorphisms (SNPs). SNP is the most frequent form of genetic variation and is a resource for mapping complex genetic traits. With the completion of the Human Genome Project, SNPs have been developed as a high-resolution marker set for accelerating the mapping of human disease genes. A major goal in animal agricultural genetics is to understand the role of common genetic variants in controlling quantitative traits. The development of high quality SNP maps for agriculturally important species can be viewed as a fundamental tool for animal breeding program. This will involve investigating the nature of gene variation in animal populations, assembling a large database of SNPs in candidate genes and studying association genetics of SNPs with particular quantitative trait and genome-wide association scans. At present, our knowledge of animal gene variation
and identification of SNPs from domestic animal genome remains rudimentary.

Growth rate is the most important area for the poultry breeding industry due to its economic value and has been improved dramatically in the past through mass selection which promises moderate to high heritability. Growth, however, is a complex process that involves both increase in mass and differentiation, and maturation of many tissues especially for skeletal muscles, therefore a number of complications (such as reduced reproductive performance, increased carcass fat, skeletal abnormalities, and ascites) have arisen with intense mass selection for high growth rate. This disturbance of physiological homeostasis has become a selection barrier to continued improvements in growth performance. As it has been demonstrated that genetic polymorphisms are associated with quantitative traits (e.g. growth rate)\(^2\), therefore it may be possible to use SNPs as genetic markers for direct genome-wide assessments in order to eliminate undesirable alleles and maximize the degree of homozygosity for desirable alleles. Although a genome-wide assessment is necessary to eliminate undesirable alleles, it may be possible to use SNPs as genetic markers for direct genome-wide assessments in order to eliminate undesirable alleles and maximize the degree of homozygosity for desirable alleles. Although a consensus linkage map of chicken genome has recently been reported\(^2\), the identification and specific characteristics of SNPs in the chicken genome are still at an early stage.

In this paper, we choose chicken myostatin gene as a candidate for searching SNPs and investigating the genetic association of SNPs with growth traits. Our results show that the SNPs are associated not only with skeletal muscle growth but also with fat deposition in chicken. These findings provide the first genetic evidence for myostatin function in regulating adipose growth, and also strongly suggest that myostatin could be used as a genetic marker in poultry breeding programs for selecting chicken with more skeletal muscle and less fat.

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**References**