IDENTIFICATION OF TWO NOVEL SINGLE NUCLEOTIDE POLYMORPHISM SITES IN THE MYOSTATIN (MSTN) GENE AND THEIR ASSOCIATION WITH CARCASS TRAITS IN MEAT-TYPE RABBITS (ORYCTOLAGUS CUNICULUS)

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Abstract: Two unknown single nucleotide polymorphism (SNP) sites in exons 1 (c.194C>T) and 2 (c.445T>A) of meat-type rabbit MSTN gene were identified in the study. Our objective was to analyse the population genetics structure of the two novel SNP sites in 230 individuals from six breeds and their associations with carcass traits of rabbits. We found that live body weight (BW), cold carcass weight (CCW), reference carcass weight (RCW), CCW percentage (P_CCW) and RCW percentage (P_RCW) of the rabbits with the genotype CC at the c.194C>T of exon 1 or AA at the c.445T>A of exon 2 were significantly higher than those with other genotypes. Diplotype significantly affected BW, RCW, CCW, P_CCW (P<0.01) and P_RCW (P<0.05). CC/AA was the advantageous diplotype for BW, RCW, CCW and P_CM, and TT/AA was the advantageous diplotype for P_CCW and P_RCW. In contrast, TT/TT was the negative diplotype for BW, CCW, RCW, P_CCW and P_RCW, and TT/AA was the negative diplotype for P_CM. The results suggest that the two new mutations of MSTN gene significantly affected BW, CCW, RCW, P_CCW and P_RCW of rabbits, and MSTN may be an important candidate gene of carcass traits in meat-type rabbits.

Key Words: rabbit, myostatin gene, single nucleotide polymorphisms, carcass traits.

INTRODUCTION

Myostatin (MSTN), or growth and differentiation factor 8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily that acts as a negative regulator of skeletal muscle development and growth in mammals (McPherron et al., 1997, 2002). MSTN can inhibit myogenic and sarcogenic cell proliferation by down-regulating the expression of MyoD, Pax and Myf25 (Rios et al., 2002; Joulia et al., 2003; McCroskery et al., 2003) and has been shown to regulate muscle development and growth by suppressing the transcription of MyoG family members (Joulia et al., 2003). Consequently, MSTN has received much attention from the meat-producing animal breeding industry as a candidate gene that can control growth and carcass traits (Bellinge et al., 2004). Some mutations disrupting MSTN function caused double-muscling phenotypes in cattle (Kambadur et al., 1997; Grobet et al., 1997, 1998; McPherron et al., 1997, 2002; Marchitelli et al., 2003) and dogs (Mosher et al., 2007). In addition, MSTN knock-out goat (Guo et al., 2016) and mouse (McPherron et al., 1997; Lin et al., 2002) performed a high growth rate in the early stage and muscular hypertrophy and hyperplasia, respectively. Associations between MSTN polymorphisms and carcass traits have been reported for numerous livestock species including pig (Li et al., 2002; Jiang et al., 2008), goat (Liu et al., 2006), cattle (Grobet et al., 1998; Guo et al., 2007), chicken (Zhu et al., 2007), duck (Lu et al., 2008) and rabbit (Fontanesi et al., 2011; Qiao et al., 2014; Abdel-Kafy et al., 2016; El-Sabrout and Aggag, 2017), but few reports have investigated the effects of different SNPs in rabbit MSTN gene on production traits.

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Rabbits exhibit several traits of economic importance as meat livestock and as a common experimental animal. More than 750 thousand tons of rabbit meat has been produced in China every year since 2010, and improving the yield of rabbit meat by genetic selection or gene modification is a highly significant aim. Therefore, the exploration of all genetic mutation sites which affect the growth of skeletal muscles has an important theoretical and practical significance.

The rabbit MSTN gene which consists of three exons and two introns is highly conserved and is expressed in developing and mature skeletal muscle (McPherron et al., 1997; Bellinge et al., 2004; Fontanesi et al., 2008, 2011; Qiao et al., 2014). MSTN knock-out rabbits exhibited increased birth weight and a significant increase in the weight ratios of the quadriceps and biceps muscles to the whole body (Guo et al., 2016). The role of MSTN in growth and carcass-related traits suggests that it may be an important genetic marker in rabbits. Fontanesi et al. (2011) identified 4 SNPs in 14 rabbits representing breeding or lines, and Sternstein et al. (2014) identified two SNPs in crossbred population of Giant Grey and New Zealand white rabbit. The present research was carried out to detect the genetic variation within MSTN gene of the rabbits from six breeds and to estimate the association of MSTN SNPs, alone and in combination, with carcass traits in the rabbits.

MATERIALS AND METHODS

Animal

A batch of 230 rabbits from six breeds (30 Harbin white, 32 Belgians, 21 Hotot, 63 Zika, 36 California and 48 Tianfu black) was obtained from the Sichuan Agricultural University research farm. All rabbits were housed and fed under the same conditions. The nutritional levels and feeding management have been addressed by Zhang et al. (2011). All experimental procedures were approved by the Animal Ethics Monitoring Committee of Sichuan Agricultural University and The use of experimental rabbits followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (3rd Ed). The approval number is SCAUS20163636.

Growth and carcass traits measurement

Live body weights (BW) of all rabbits were measured after 12h starvation at 135 d of age, while cold carcass weight (CCW) and reference carcass weight (RCW) were measured after slaughter at the age of 135 d according to the description from George et al. (2015). Then, the CCW and RCW percentages were calculated according to the formula: $P_{CCW} = (CCW/BW) \times 100\%$ and $P_{RCW} = (RCW/BW) \times 100\%$, respectively. To measure the cooked meat percentage ($P_{CM}$), 100 g right-leg semi-membranous muscle was collected 2 h post-slaughter, weighed ($W_0$) and steamed at 100°C for 45 min, cooled at room temperature for 30 min and weighed again ($W_1$). The cooked meat percentage (CMP) was calculated according to the formula: $P_{CM} = (W_1/W_0) \times 100\%$ (Naveen et al., 2016).

Sampling and DNA extraction

Genomic DNA was isolated from 1.5 mL blood by standard phenol-chloroform extraction. The integrity and concentration of genomic DNA were assessed by 0.8% agarose gel electrophoresis and photometry using Gene Quant II (GE, USA), respectively. The purity of genomic DNA met the experimental requirements. Genomic DNA was diluted to a concentration of 20 ng/μL and stored at −80°C until polymerase chain reaction (PCR) amplification.

PCR amplification, PCR-Single Strand Conformation Polymorphism analysis (SSCP) and Sequencing

All primers (Table 1) were designed according to their DNA sequence, containing two exons (AM931155; AM931156) of the MSTN gene in GenBank database of NCBI, using Oligo 6.0 software (http://www.oligo.net/downloads.html) and synthesised by Invitrogen Shanghai Company (Shanghai, China). PCR amplification was performed in 10 μL reactions containing 5.0 μL of 2×PCR MasterMix from TaKaRa (Dalian, China), 0.4 μL of 10 pmol/μL forward and reverse primer, respectively, 0.7 μL 25 ng/μL template DNA and 3.5 μL RNase free water. Samples were amplified in a PTC-100 thermal cycler (MJ Company, USA) by an initial denaturation at 96°C for 10 min, 35 cycles of denaturation (95°C, 30 s), annealing (see Table 1 for respective temperatures, 30 s) and extension (72°C, 15-30 s), and a final extension at 72°C for 5 min.
Two novel SNPs of MSTN are associated with carcass

To confirm the reliability of SNP detection, all PCR products from 230 individuals were sequenced in two
directions by Sanger method after the amplification of products of MSTN gene was conducted in 12% non-
denaturing polyacrylamide gel to determine single strand conformation polymorphism (SSCP) (Shi et al.,
2009). The sequences were compared by BLASTn searching in GenBank and analysed by DNAMAN Sequence Analysis
Software (Lynnon Corporation, St-Louis, Pointe-Claire, Quebec, Canada). RNA structure fold was predicted by online
tool (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/) to explain the potential effect of two SNPs on
carcass traits.

Statistics

Data of P\textsubscript{CCW}, P\textsubscript{RCW} and P\textsubscript{CM} were not normally distributed by Shapiro-Wilk test (Meredith et al., 2006) and the
percentage data required arcsine transformation before further processing. The transformed data and BW, CCW
and RCW were analysed by the general linear model (GLM) procedure of SAS (SAS Inst. Inc., Cary NC). The genetic
effects were assessed by a mixed procedure according to the following GLM:

\[ Y = \mu + B + G + bX + e, \]

where \( Y \) was the
dependent variance; \( \mu \), the population mean; \( B \), the fixed effects of the breed; \( G \), the fixed effects of the genetic
(genotype or diplotype) background; \( X \), the growth and carcass traits; \( b \), the coefficient of regression; and \( e \), random
error. Multiple comparisons were performed by least squares means (LSM) analyses. Differences were considered
significant (\( P<0.05 \)), and all values are presented as the mean±standard deviation. Chi square test was performed in
Hardy-Weinberg equilibrium of the frequency of alleles and genotype.

RESULTS

Single Nucleotide Polymorphisms (SNPs) in exons of the rabbit MSTN gene

The sequence data revealed two new SNPs which were localised in exon 1 and 2 (Figure 1). There was a C\textrightarrow{}T
mutation at position c.194 (relative to GenBank accession No: AM931155) in exon 1, and a T\textrightarrow{}A mutation at position
c.445 (relative to GenBank accession No: AM931156) in exon 2. Both SNPs were synonymous mutations that did not
change the amino acid sequence of MSTN.

Figure 1: Structure of the rabbit myostatin gene and positions of mutations. We found the two novel SNPs are c.194C>T
and c.445T>A, listed below the schematic gene structure map, and six SNPs (125T>C, 108C>T, 374+234G>A,
713T>A, 713+34C>T and 3'UTR 194A>G) which have been reported were marked on the schematic map of gene
structure. The sequenced regions comprise the exons and 5' and 3' exon flanking regions.
**Association of the MSTN alleles and genotypes with rabbit carcass traits**

The frequencies of the alleles and genotypes are summarised in Table 2. In exon 1, allele C was predominant over allele T in all six rabbit lines, and the homozygous genotype CC had the highest frequency in all populations. In exon 2, the heterozygous TA genotype had the highest frequency in all populations. The frequency of alleles and genotypes did not deviate from the Hardy-Weinberg equilibrium (HWE) in all six rabbit breeds by chi-square ($\chi^2$) test, with the exception of exon 1 in Zika rabbits.

The associations between MSTN genotypes and carcass traits (BW, CCW, RCW, $P_{CCW}$, $P_{RCW}$, $P_{CM}$) were assessed by GLM analysis (Table 3). In exon 1, genotype significantly affected BW, CCW, RCW, $P_{RCW}$ and $P_{CCW}$. In particular, the BW, CCW, RCW, $P_{RCW}$ and $P_{CCW}$ of these rabbits with the CC genotype was significantly higher than those with the TT genotype ($P<0.05$). The BW, CCW and RCW of CC rabbits was significantly higher than those of CT rabbits ($P<0.05$).

In exon 2, genotype strongly influenced the CCW, $P_{RCW}$ and $P_{CM}$. Rabbits of the AA genotype had significantly higher CCW, $P_{RCW}$ and $P_{CM}$ ($P<0.05$) than those of the TT genotype. The results suggested that CC was the advantageous genotype for BW, RCW, CCW, $P_{RCW}$ and $P_{CCW}$, whereas TT was the negative genotype for BW, CCW, RCW, $P_{CCW}$, $P_{RCW}$ and $P_{CM}$.

**Construction of haplotypes and their associations with rabbit carcass traits**

Nine diplotypes (Table 4) were obtained based on the six haplotypes in Table 2. Among them, the frequency of the diplotype CC/AA was the highest (31.74%) and the frequency of the diplotype TT/AA was the lowest (0.87%). Mixed model analysis showed that the MSTN diplotype strongly correlated with carcass traits and significantly affected the BW, RCW, CCW and $P_{RCW}$ ($P<0.01$) and the $P_{CCW}$ and $P_{CM}$ ($P<0.05$). The CC/AA diplotype was advantageous for BW, RCW, CCW and $P_{CM}$, whereas the TT/AA diplotype was negative for BW, RCW, CCW, $P_{RCW}$ and $P_{CCW}$, and the TT/TT diplotype was negative for BW, RCW, CCW, $P_{RCW}$ and $P_{CM}$.

**Discussion**

The identification of genetic variations in MSTN is of great interest to animal breeding, as a better understanding of the effects of MSTN on growth and carcass traits offers a potential genetic marker to improve meat production (Joulia-Ekaza et al., 2007). In the present study, two SNP loci were first identified. At position c 194, there was a C to T transversion in exon 1 and a T to A transversion at position c 445 in exon 2. The two mutations were synonymous. Previous studies had identified a T to A transversion in exon 2 of rabbit MSTN gene (Fontanesi et al., 2011; Abdel-Kafy et al., 2016), and Li et al. (2002) identified a G to T transversion in exon 2. Moreover, an A to G

| Locus | Line  | n   | CC  | CT  | TT  | C    | T    | Pr   |
|-------|-------|-----|-----|-----|-----|------|------|------|
| Exon 1|       |     |     |     |     |      |      |      |
|       | Harbin| 30  | 0.63| 0.27| 0.10| 0.767| 0.233| 0.969|
|       | Hotot | 21  | 0.62| 0.38| 0.00| 0.810| 0.191| 0.973|
|       | Tianfu| 48  | 0.60| 0.31| 0.08| 0.761| 0.240| 0.991|
|       | Belgian| 32  | 0.66| 0.28| 0.06| 0.797| 0.203| 0.992|
|       | Zika   | 63  | 0.81| 0.14| 0.05| 0.881| 0.119| 0.943*|
|       | California | 36   | 0.47| 0.39| 0.14| 0.667| 0.333| 0.992|
| Exon 2|       |     |     |     |     |      |      |      |
|       | Line   | n   | TT  | TA  | AA  | T    | A    |      |
|       | Harbin| 30  | 0.37| 0.50| 0.13| 0.617| 0.383| 0.998|
|       | Hotot | 21  | 0.29| 0.62| 0.10| 0.595| 0.405| 0.963|
|       | Tianfu| 48  | 0.27| 0.40| 0.33| 0.469| 0.531| 0.981|
|       | Belgian| 32  | 0.22| 0.53| 0.25| 0.484| 0.516| 0.998|
|       | Zika   | 63  | 0.17| 0.54| 0.29| 0.444| 0.556| 0.995|
|       | California | 36   | 0.22| 0.58| 0.19| 0.514| 0.486| 0.986|

n: number of rabbits per breed; Pr is the probability of $\chi^2$-test for the Hardy-Weinberg equilibrium. *Mean the frequency of alleles and genotypes deviate from the H-W equilibrium (HWE).
Two novel SNPs of MSTn are associated with carcass

**Table 3:** GLM analysis of associations between rabbit carcass traits and myostatin gene SNPs.

| Traits | Exon 1 | Exon 2 |
|--------|--------|--------|
|        | CC (149) | CT (63) | TT (18) | TT (57) | TA (117) | AA (56) |
| BW (kg) | 2.480±0.024<sup>a</sup> | 2.378±0.037<sup>a</sup> | 2.316±0.070<sup>b</sup> | 2.424±0.040 | 2.419±0.028 | 2.498±0.040 |
| CCW (kg) | 1.171±0.015<sup>a</sup> | 1.096±0.023<sup>a</sup> | 1.049±0.043<sup>ab</sup> | 1.114±0.024<sup>ab</sup> | 1.130±0.017<sup>b</sup> | 1.191±0.025<sup>ab</sup> |
| RCW (%<sub>P</sub>) | 1.128±0.038<sup>a</sup> | 1.051±0.029<sup>ab</sup> | 0.992±0.026<sup>b</sup> | 1.065±0.036 | 1.085±0.032 | 1.149±0.035 |
| P<sub>CCW</sub> (%) | 47.5±0.4<sup>a</sup> | 46.4±0.5<sup>b</sup> | 45.2±1.0<sup>ab</sup> | 46.1±0.6<sup>a</sup> | 46.9±0.4<sup>ab</sup> | 48.0±0.6<sup>ab</sup> |
| P<sub>CCW</sub> (%) | 51.7±0.4<sup>a</sup> | 50.3±0.6<sup>b</sup> | 49.1±1.1<sup>ab</sup> | 50.1±0.6 | 51.2±0.5 | 51.8±0.7 |
| P<sub>CM</sub> (%) | 62.4±0.007 | 63.0±1.0 | 59.5±2.0 | 59.5±1.1<sup>b</sup> | 62.9±0.7<sup>b</sup> | 63.4±1.1<sup>ab</sup> |

BW: live body weight at 135 d; CCW: cold carcass weight; RCW: reference carcass weight; P<sub>CCW</sub>=(CCW/BW)×100%; P<sub>CM</sub> cooked meat percentage. *<sup>a</sup>The least square means with different superscripts indicates significant differences (<<sup>b</sup>P<0.05) among different genotypes within exon 1 or 2. P superscript represents the positive genotypes in exon 1 and 2, and N superscript represent the negative genotypes in exon 1 and 2.

Two novel SNPs of synonymous mutations in exon 1 of MSTN (Li et al., 2002) and a conversion of G to A at position 84 in exon 1 of MSTN in German Merino sheep were examined by Zhang et al. (2007). These variations in MSTN gene from rabbit and other animals were all synonymous mutations. These studies suggest that MSTN has multiple SNP sites which often result in synonymous mutations. Synonymous single base mutation could not change the amino acid sequence of MSTN, but affected the modification or the folding of mRNA and thus resulted in altered biological functions (Simon et al., 2002). The findings are further supported by our present study, which demonstrated that c.445T>A SNP resulted in a significant change in RNA secondary structure of exon 2. Moreover, the mutation caused the minimum free energy of RNA secondary structure to change from −75.2 kcal/mol to −72.8 kcal/mol, which affected the secondary structure stability of RNA and might affect the subsequent protein translation process. However, the c.194C>T SNP did not change the RNA secondary structure of exon 1, although the minimum free energy changed from −92.8 kcal/mol to −93.6 kcal/mol (Figure 2). Therefore, synonymous MSTN mutations may affect the biological phenotype, especially in terms of carcass traits.

Two SNPs of synonymous mutations in exon 1 of MSTN significantly impacted carcass weight, abdominal fat weight and breast muscle weight of chickens (Zhu et al., 2007), and an exon 1 SNP in MSTN of Steppe red cattle and hybrid cattle (Limousin cattle×grassland red bull) had significant associations with the daily gain and dressing percentages (Guo et al., 2007). An SNP (c.476T>A) in the 5′ regulatory region of rabbit MSTN resulted in increased liver weight.

**Table 4:** Association between myostatin diplotype and growth and carcass traits.

| Diplotype | Fr. (%) | BW (kg)<sup>a</sup> | RCW(kg)<sup>**</sup> | CCW (kg)<sup>**</sup> | P<sub>RCW</sub> (%)<sup>**</sup> | P<sub>CCW</sub> (%)<sup>*</sup> | P<sub>CM</sub> (%)<sup>*</sup> |
|-----------|---------|----------------|----------------|----------------|----------------|----------------|----------------|
| CC/TT     | 15.65   | 2.463±0.050 | 1.099±0.028 | 1.126±0.030 | 46.0±0.7 | 50.0±0.8 | 59.7±1.4 |
| CC/TA     | 17.39   | 2.472±0.035 | 1.093±0.026 | 1.172±0.021 | 47.7±0.5 | 52.2±0.6 | 62.9±1.0 |
| CC/AA     | 31.74   | 2.511±0.047 | 1.142±0.012<sup>a</sup> | 1.208±0.029<sup>a</sup> | 48.4±0.7 | 52.2±0.8 | 63.6±1.3<sup>ab</sup> |
| CT/TT     | 6.09    | 2.413±0.079 | 1.072±0.034 | 1.141±0.048 | 47.5±1.1 | 51.4±1.3 | 59.1±2.2 |
| CT/TA     | 15.22   | 2.327±0.050 | 1.003±0.013 | 1.061±0.031 | 45.8±0.7 | 49.8±0.8 | 63.6±1.4 |
| CT/AA     | 6.09    | 2.470±0.079 | 1.081±0.040 | 1.140±0.048 | 46.6±1.1 | 50.4±1.3 | 63.9±2.2 |
| TT/TT     | 3.04    | 2.246±0.112<sup>ab</sup> | 0.892±0.026<sup>ab</sup> | 0.994±0.068<sup>a</sup> | 43.8±1.6<sup>ab</sup> | 48.1±1.8<sup>ab</sup> | 59.2±3.1 |
| TT/TA     | 3.91    | 2.344±0.099 | 1.031±0.027 | 1.056±0.060 | 45.2±1.4 | 48.9±1.6 | 60.7±2.7 |
| TT/AA     | 0.87    | 2.438±0.210 | 1.068±0.010 | 1.208±0.128 | 49.7±3.0<sup>a</sup> | 53.4±3.4<sup>ab</sup> | 55.1±5.8<sup>a</sup> |

BW: live body weight at 135 d; CCW: cold carcass weight; RCW: reference carcass weight; P<sub>CCW</sub>=(CCW/BW)×100%; P<sub>CM</sub> cooked meat percentage. *<sup>a</sup>P superscript represent the advantageous diplotypes, and N superscript represent the negative diplotypes.

All values are presented as the least squares mean±standard deviation. *<sup>P<0.05, **P<0.01.</sup>
carcass weight, forelegs weight, back and waist weight, ham weight and tare weight (Qiao et al., 2014), and Abdel-Kafy et al. (2016) also found that rabbit with the MSTN genotype (194GG) correlated with the highest values in BW and daily weight gain, and allele T at the c747+34c>T SNP in intron 2 was significantly associated with increased BW, whereas no significant effects were found for c.-125T>C and c.747+34C>T in the coding region. In the present study, we also confirmed that the new SNPs in exon 1 and exon 2 of MSTN have significant associations with rabbit carcass traits.

The total effect of two variable sites on carcass traits was analysed by haplotype analysis. The results showed that individuals with the CC/AA or TT/AA diplotype had higher BW, RCW and CCW or P_RCW and P_CCW than the other seven diplotypes. The frequency of the CC/AA diplotype was highest among all diplotypes, and it was the advantageous diplotype for BW, RCW and CCW, suggesting that BW, RCW and CCW may be preferential factors in the genetic selection of rabbits. Therefore, the SNPs in exon 1 or exon 2 of MSTN may be used as a potential site for BW, RCW and CCW selection. The absence of HWE deviation and lower than 50% frequency of advantageous diplotypes (CC/AA: 31.74%, TT/AA: 0.87%) suggest that genetic selection of MSTN still has great potential for improving the BW, RCW and CCW or P_RCW and P_CCW.

Figure 2: The effect of c.194C>T and c.445T>A mutations on RNA secondary structure of exon 1 and exon 2 of myostatin (MSTN). A, B: The secondary structure of the MSTN exon 1 of different alleles; C, D: The secondary structure of the MSTN exon 2 of different alleles. Arrows: Mutant site.
Two novel SNPs of MSTN are associated with carcass

In conclusion, we found two novel variations in exon 1 (c.194C>T) and exon 2 (c.445T>A) of the MSTN gene and they significantly affect rabbit carcass traits. The advantageous diplootypes might be used in genetic selection for improving BW, RCW and CCV or P_{BW}, P_{RCW} and P_{CCV}. Further population-wide studies are required to test the association of the two SNPs with carcass traits, and the stable verification of the SNPs and advantageous diplootypes in different generations may also be necessary.

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