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Identification of Myostatin Gene Polymorphism in Padjadjaran Sheep by Using RT-PCR Method

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Abstract: The aims of this research was to identifying myostatin gene (MSTN) polymorphism and the it is association with body weight, sex and birth weight in Padjadjaran sheep, blood samples were collected from the jugular vein 3 ml of each head, used kit DNA genomic samples of whole blood 2 ml DNA isolation, to identify candidate gene used the Ensenbl database, three pairs of primers were designed to amplify exons 1, 2 and 3 of MSTN gene. The 25 µl volume contained 50 ng of genomic DNA, 12.5 µl 2x Reaction mix, 0.5 µM of each primer, and 0.5 units of Taq DNA polymerase. The cycling protocol was 5 min at 95°C, 30 cycles of denaturation at 94°C for 30 second, annealing at 58-68°C for 1 second and extended at 72°C for 1 second, with final extension at 72°C for 3 second. All samples sent to Malaysia for sequencing and used BioEdit program to identify nucleotide substitution and sequenced results compared with MSTN gene reference coding regions. Calculation of genotypes, allele's frequencies, mean expected and observed heterozygosities, and Chi-square test was performed. Body weight and body measurements were used for statistical analysis by GLM procedure and the LSD (SAS version 9.0). Results showed that exon 1 of c.101G>A position was dimorphic and c.120insA genotype was monomorphic, and c.960delG locus was polymorphic with genotype frequencies of GG 96.872% and GC 5.128% respectively this locus did not show Hardy-Weinberg equilibrium ($P < 0.05$). SNP is an AAG →TCG and AAG → GCG substitutions allocated in the 3rd exon, these variations only detected in two individuals were found to be heterozygous for the c.960delG. An association was revealed between c.960delG, gender and birth weight were ($p = 0.205$) and ($p = 0.042$) respectively, and body weight ($P>0.001$). It can be concluded that, although MSTN polymorphism effect on body weight, birth weight and sex, further study needs to be conducted and involving many more sheep of Padjadjaran breed including the other Indonesian sheep to confirm these observations.

Key words: identification, MSTN gene, Padjdjaran sheep and RT-PCR

1. Introduction

Recent scientific evidence suggests that myostatin (a member of the transforming growth factor TGF-β superfamily, which is encoded by the MSTN gene) regulates skeletal muscle development in a range of mammalian species, including the horse [1]. The MSTN gene is composed of three exons and two introns. Myostatin protein is synthesized as a precursor, and upon proteolytic processing gives an N-terminal latency-associated peptide (myostatinpropeptide or LAP-fragment) and a smaller

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mature peptide at the C-terminus [2]. Myostatin is produced primarily in skeletal muscle cells, circulates in the blood, and acts on muscle tissue by binding to a cell-bound receptor, activin type II. It regulates both the number and growth of muscle fibers, and inhibits muscle differentiation and growth [1]. Nowadays, through the availability of sheep whole genome sequence, the genomic selection can be implemented in sheep breeding programs to increase meat production traits. The genetic gain rate for the double-muscling trait depends not only on allelic frequency but also on the proportion of homozygote animals for the A allele in the population, due to the partially recessive action of myostatin on the muscle phenotype. Consequently, selection of this SNP could be substantially beneficial for sheep breeders [3]. The number of muscle fibers largely determines the amount of postnatal muscle growth, the total count of muscle fibers typically remains the same in postnatal muscle growth [4].It has been suggested that there might be an increase in fiber numbers shortly after birth [5].

2. Materials and Methods

2.1 Animals of Experiment and Management

Data collected from total (50 heads) of Padjdajaran sheep, Lambs were tagged and identified to their dams, and were born as (n =14) singletons, (n = 20) twins, (n = 5) triplets. These lambs were reared under extensive feeding system and grazing. Parameters determined in this study are body weight (BW) at six month of age and birth weight, litter size and sex. Blood samples were collected from the jugular vein 3 ml of each lamb into EDTA [10 mL EDTA (spray dried), 10.8-mg Vacutainer tubes]. All samples delivered back to the laboratory of faculty of medicine in an ice box.

2.2 Genomic DNA Extraction

Used kit DNA genomic samples of whole blood 2ml with anti-coagulation EDTA. Added 300 µl bloods to Eppendorf tube 1.5 ml and 900 µl (3 times volume blood) 1x RBC Lysis solution. Invert the tube 2-3 times during preparation. Incubate the samples for at least 10 minutes at room temperature. Centrifuged at 1300 rpm for 20 sec, removed the supernatant and leave it until the leucocyte pellet of white blood cells appeared, then vortex the pellet to spread the cells into the remaining drops of supernatant. Added 300 µl RBC Lysis solution to those tubes contained pellet and pipetted it up and down to lyse the cells and 1.5 µl of RNAs,then incubated at 37°C for 15 sec. Added 100 µL protein precipitation solution and vortexes for 15 second after that centrifuged for 3 minute and moved the supernatant to new Eppendorf tube. Added 600 µL is opropanol to the supernatant to accumulate the DNA. Mixed the DNA with up and down-stirring about 10 times by hand and centrifuged one minute to appear DNA pellet then, separated the DNA from the supernatant and washed with alcohol 70% and dried it by (Concentrator 5301 device). DNA pellet resolved with 50 µL TE buffer and conserved in a freezer at -20°C until using.

2.3 Identification ofMSTN Gene

Used the chromosome regions that have been located, to identify candidate genes using the Ensenbl database (www.ensembl.org) Candidate gene was chosen based on their known function or potential involvement with muscle development.

2.4 PCR Conditions

According to (UCSC genomic browser), two pairs of primers were designed to amplify exons 1 and 3 of MSTN gene and screened for polymorphisms. The 25 µl volume contained 50 ng of genomic DNA, 12.5 µl 2x Reaction mix (including 500µMdNTP each; 20mMTris-HCl, pH 9; 100mMKCl; 3mM MgCl2), 0.5µM of each primer, and 0.5 units of Taq DNA polymerase [6]. The cycling protocol was 5 min at 95°C, 30 cycles of denaturing at 94°C for 30 s, annealing at 58-68°C for 1second and extending at 72°C for 1sec, with final extension at 72°C for 3 second.

Table 1 Primer forward and primer reverse.

Amplified fragment	Size bp	Primer name	Primer sequence	Tm
MSTN	496	Foreward:	GGCAGGCATTAACGTTTGG	61.4°C
		Reverse:	AGTCGTTGCTCTGCCCTAGC	62.0°C
MSTN	484	Foreward:	AGATTGACATGGAGGCGTTC	60.1°C
		Reverse:	CAAGGTTTTTAGCATGTTATTTTCAG	59.6°C
MSTN	487	Foreward:	TGCGGTAGGAGAGTGTTTGG	61.2°C
		Reverse:	AAAATTGTTGAGGGGAAGACC	59.3°C

Source: UCSC genomic browser.

2.5 Sequencing

All samples sent to Malaysia for sequencing and used BioEdit program to identify nucleotide substitution and sequenced results compared with MSTN gene reference coding regions. For DNA Sequencing samples preparation and quantification used UV Quantification by UV Spectrometry 25 μL for each sample, and purified by PCR Clean-up kit.

2.6Data Analysis

Calculation of genotypes, allele's frequencies, mean expected and observed heterozygosities, and Chi-square test was performed. Body weight and body measurements were used for statistical analysis by GLM procedure and the "LSD" (software SAS program version 9.0)in order to determine the correlation between MSTN genotype and body weight, gender and litter size described by Shi and He L. (2005) [7].

3. Results

There are only three variations reported in the ovine MSTN coding region, these include: c.101G > A which results in a mis-sense substitution of Glutamic acid →Glycine at amino acid 34 (aa34/codon 34/c34) (Zhou et al., 2008); c.120insA which results in a premature stop codon at aa49 and leads to a completely nonfunctional MSTN due to the bioactive carboxy-terminal end of the protein not being produced [8] and c.960delG which results in a disruption of the reading frame from aa320 to a pre-mature stop codon at aa359 [9]. Genotyping of the three MSTN positions, c.960, c.101G>A and c.120insA were carried out as described. First, the animals were genotyped only at c.101G>A position, and retyped at c.120insA and c.960 position. Results showed that exon 1 of c.101G>A position was dimorphic and c.120insA genotype was monomorphic, and all samples showed the homozygotus genotype only one sample in c.101G>A locus was genotyped as heterozygous. Different conformations were found in exon 3, G and C alleles were detected with genotype frequencies of GG 95% and GC 5% respectively, this locus did not show Hardy-Weinberg equilibrium ($P < 0.05$). Observed heterozygous for this locus was very low, showing high level of homozygosity, see Table 2. The molecular basis of the analyzed SNP is an AAG →TCG and AAG → GCG substitutions, allocated in the 3rd exon of the MSTN gene at 320th codon, these variations only detected in two individuals lambs were found to be heterozygous for the c.960delGlocus and 37 lamb heads were homozygous at 3rdexon. Also detected only one individual variation at 1st exon of c.101G>A genotype which results in a mis-sense substitution of Glutamic acid →Glycine at amino acid 34as reported by Zhou et al. (2008) [10].

An association was revealed between MSTN c.960delG genotype and gender ($p = 0.205$) was shown at Table 3, MSTN c.960delG presence and birth weight of type of birth. Those lambs with the c.960delG GG genotype ($n = 37$) had a mean of birth weight is 2.34±0.05 kg, while lambs with the c.960delG GC genotype ($n = 2$) had a mean birth

weight of 3.6±0.4 kg, males heavier than females but there is no significant different. Generally birth weight of this study ranged 1.5 (twin) to 4 kg (single), the overall means of birth status of single, twins and triplets were 2.68±0.051, 2.20±0.046 and 1.97±0.082 kg respectively for GG lambs, and 3.6±0.4 kg (single) while the type of birth was significantly different and (p = 0.042). Lambs with MSTN GC genotype (n = 2) has overall means of body weight 14.80 however while lambs with GG genotype (n = 37) is 11.39, body weight was highly significant different.

Table 2 Frequencies of genotypes/alleles in studied sheep.

	MSTN	Genotype	Frequency	Allele	Frequency
Observed	GG	GC	CC	G	C
c.960delG	0.95	0.05	-	0.975	0.025
c.101G>A	-	1	-	-	-
c.120insA	-	-	-	-	-
predicted	0.9	0.097	0.003		

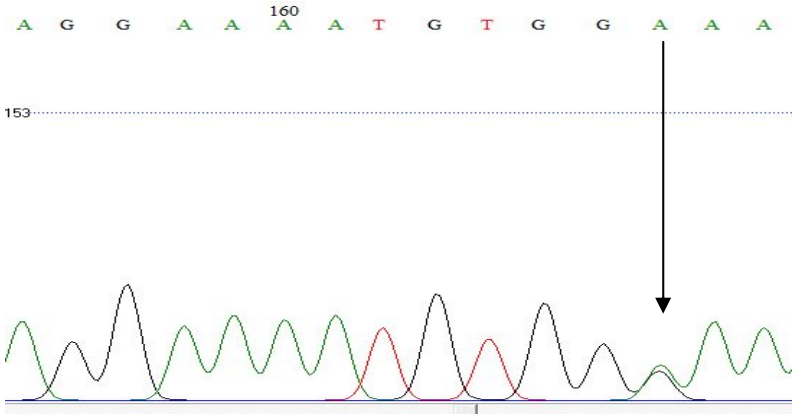

Fig. 1 The results of DNA sequencing (Heterozygotic Genotype). The Arrow Indicates the SNP Located in the 34th Codon in the 1st Exon of the Sheep MSTN Gene.

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....|....| ....|....| ....|....| ....|....| ....|....|
 205  215       225   235    245
refmTTATTGCACC TAAAAGATAT AAGGCCAATT ACTGCTCTGG AGAATGTGAA
samp   TTATTGCACC TAAAAGATAT AAGGCCAATT ACTGCTCTGG AGAATGTGAA
samp   TCATTGACCG TAAAAGATAA AAGGTTAGTT TGGGCTCGGG ATAATGCGAC
samp   TTATTGCACC TAAAAGATAT AAGGCCAATT ACTGCTCTGG AGAATGTGAA
samp   TTATTGCACC TAAAAGATAT AAGGCCAATT ACTGCTCTGG AGAATGTGAA
Clust* ****     *********  ****  * **    **** ** * ** * **
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Fig. 2 Alignment of the sheep splice variants of Myostatin Gene, used BioEdit program, underline nucleotide indicate to substitutions.

Table 3 Least significant differences of sex, type of birth and body weight.

Sex	GG	CG	type of birth	GG	GC	body weight at (6 month)	
Male	2.41±0.043	4.0	single	2.68±0.051	3.6±0.40	GG	GC
Female	2.30±0.49	3.2	twin	2.20±0.046	-	11.39 kg	14.80 kg
Mean	2.34±0.05	3.6±0.40	Triplet	1.97±0.082	-		

4. Discussion

This is the first study to report identification of MSTN in padjadjaran sheep, Sumantri et al. (2008) and Dagong et al. (2011) reported that MSTN c.del960G locus in Indonesia local sheep is very low [11, 12]. This is indicated by the value of one genotype frequency and allele which has a value of 1, which marks the fixation process, this value is lower they used CAST|MspI and CAST loci at 6th exon in Indonesian local sheep respectively. The absent of deletion in 1-bp deletion at MSTN c.del960G can be caused by tropical adaptation process which suggested that the animal which can survive in this environmental are having small performance. In this case, the presence of the c.960delG mutation in both alleles means that no functional myostatin protein is expected to be produced [9]. If myostatin not to be expressed, so the negative growth regulation will failure and will increase the number of muscle fibers (hyperplasia).But in this study we detected polymorphisms in this gene (GC and GG) in three individual's local sheep at 1st and 3th exon but this result not in agreement with Sumantri et al. (2008) [11].

An association between MSTN c.del960G genotype and gender ratios in Padjadjaran sheep west java, a result that is unexpected. Myostatin is primarily expressed in skeletal muscle, but it is not restricted to this tissue. MSTN has also been detected in the heart muscle of the mouse and sheep [13] and mammary glands of pigs [14]. In chicken embryos, myostatin expression can be detected as early as the blastoderm stage [15]. In addition, myostatin is also involved in human maternal/foetal nutrient partitioning by acting as a paracrine regulator [16]. In light of these reports, we suggest that MSTN might also affect other physiological activities during embryonic development, which may explain the abnormal gender ratio observed here. The actual mechanism by which this could occur is however unclear. Furthermore, in transgenic mice with muscle-specific "over expression" of myostatin, muscle mass differences were only detected in male progeny and not females [17]. Soeparna et al. (2014) reported that birth weight of this local sheep ranged between 1.8 to 3 kg, this agreed with result of birth weight of homozygote lambs, but birth weight of heterozygotus lambs were heavier and significantly different [18]. Rastogi (2001), who reported significant effects of type of birth on lamb's weight at birth, weaning and 6 months of age [19]. The birth weight advantage of single born lambs over the multiple born lambs may be due to competition for nutrient and uterine space. The multiple born lambs may have demonstrated compensatory growth after weaning. Generally, Birth year causes vacillations over body weight in different ages by the effect of climatic condition (rate of rainfall, humidity and temperature), environmental and management conditions. Climate and environmental changes have effect on the quality and quantity of pasture forages, which also affect the provision of food and other requirements for animals. The main effect will show on amount of milk production (increase or decrease). It has a direct influence on weaning weight and average daily gain from birth to weaning and has an indirect effect on birth weight due to changes in dam's environment and difference in feeding in the last weeks of pregnancy at different years [20].

5. Conclusion

It can be concluded that, although MSTN polymorphism effect on body weight, birth weight and sex, further study needs to be conducted and involving many more sheep of Padjadjaran breed including the other Indonesian sheep to confirm these observations.

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