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Investigation of GDF8 Gene Promoter in Iranian Sheep

Research Article

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ABSTRACT

Myostatin is a growth factor belonging to the TGFß superfamily. TGFß growth factors are active in the regulation of embryonic development and in tissue homeostasis in adults. Myostatin is a growth factor controlling proliferation of myoblasts in embryonic development. Mutations in coding sequences of the bovine myostatin (GDF8) gene lead to muscle hyperplasia suggesting its inhibitory function on myoblast proliferation. In bovines, loss of this gene activity has been associated with expression of the double-muscled phenotype observed in some European cattle breeds. Myostatin gene polymorphism has also been studied in sheep. Due to the role of the myostatin gene in muscle development, the objective of this study was to sequence the myostatin gene promoter and its probable mutations, which have the potential to alter myostatin gene expression. DNA from blood samples of fifteen Arabic and fifteen Kordi sheep were extracted and used to amplify a 1034bp fragment in the myostatin gene. Three mutations were observed in the myostatin gene promoter at positions 430, 450 and 530.

KEY WORDS Arabic and Kordi sheep, GDF8, myostatin gene, PCR, promoter.

INTRODUCTION

Growth and differentiation factors (GDF8) specifically regulate tissue and cell growth as well as differentiation. One of the GDF proteins, GDF8, also called myostatin (MSTN) has been considered as a novel and unique regulator of muscle growth (McPherron *et al.* 1998; Arthur *et al.* 1998; Cieslak *et al.* 2003). The MSTN gene is highly conserved across species and is expressed both in developing and mature skeletal muscle (Fahrankrug, 1999). Apart from in skeletal muscle GDF8 expression was found in postnatal adipose tissue of cattle and mice (McPherron *et al.* 1998) and in pigs' mammary glands (Ferrel *et al.* 1999). As the myostatin protein has been found in blood plasma, it is very possible that myostatin receptors are localized in various tissues (Casas *et al.* 2000). In the extracellular environment, myostatin protein forms dimers that cleave into biologically active forms (Grobet *et al.* 1998).

The muscular structure of myostatin protein is conserved across vertebrates so that their C-terminal sequences are 100% identical in mice, rats, humans, pigs and chickens (McPherron and Lee, 1998). In muscle development myostatin prevents excessive proliferation of myoblasts (Belling *et al.* 2005). Therefore mutations disrupting the function of this protein result in the formation of supernumerary muscle fibers, a phenomenon called muscle hyperplasia (McPherron *et al.* 1997; Grobet *et al.* 1998). It is common in Belgian Blue, Piemontese and Charolais cattle breeds, where a 20-25% increase in muscle mass is observed. Interestingly, this phenomenon is accompanied by a reduction of intramuscular fat, connective tissue and some internal organs. Induced mutation of murine GDF8 gene, leading to disruption of this protein structure and subsequent loss of biological activity, resulted in a 200-300%

increase in the mass of individuals' skeletal muscle (McPherron and Lee, 1998). Interestingly muscle hyperplasia in mice was accompanied by hypertrophy (increased diameter of muscle fibers); however in cattle only muscle hyperplasia was observed (Kambadur *et al.* 1997). Some studies suggested that the experimentally diminished expression of GDF8 resulted in muscle hypertrophy while increased expression resulted in muscle hyperplasia. Muscle composed of a higher number of muscle fibers per unit area possesses favorable properties such as tenderness. Moreover, a higher number of muscle fibers (hyperplasia) accounts for better and more efficient growth of lean muscle mass (Karim *et al.* 2000). The breed Sanjabi is a common breed of sheep in Iran, especially in Kermanshah province.

The objective of this study was to analyze a coding region containing mutation that potentially alters myostatin gene expression. DNA from blood samples of 150 Sanjabi sheep was extracted and used to amplify a 337bp fragment in GDF8. Restriction fragment length polymorphism (RFLP) of the PCR product was performed by an addition of the *HaeIII* enzyme to the completed PCR reaction. PCR-RFLP genotypes were analyzed. Genotype frequencies of MM, Mm and mm were detected as 2.00%, 1.33% and 96.70% respectively. Data from this study indicated that the Sanjabi breed of sheep was polymorphic for the MSTN gene, although it was not at the level of Hardy-Weinberg equilibrium (Sofi *et al.* 2010).

MATERIALS AND METHODS

Genomic DNA was extracted from blood samples of Kordi and Arabic sheep using the guanidin thiocyanate method (Boom *et al*. 1989). Identification of SNPs in the *Ovis aries* promoter used approximately 1034 bp promoter region (Gen Bank accession no. AY.918121).

The primer was designed to cover the MSTN promoter region (Table 1). The PCR reaction was performed in a 25µL reaction containing 50 ng genomic DNA, 200 µ*M* dNTP, 1.5 m*M* $MgCl_2$, 10 pmol of each primer and 1U Taq DNA polymerase. Amplification conditions were 94 ˚C for 5 min followed by 35 cycles (each cycle constituted 94 ˚C for 1 min, 63 ˚C for 1 min and 72 ˚C for 1.30 min and a final extension 10 min at 72 ˚C in a DNA thermo cycler). Electrophoresis was performed to detect the PCR product. These PCR amplified products were directly sequenced with ABI 3730 sequencer. Finally PCR product of the MSTN gene promoter was analyzed by promoter analyzer software.

RESULTS AND DISCUSSION

In this study, 1034 bp of MSTN promoter gene was sequenced (Gen Bank accession no. AY.918121) and 3

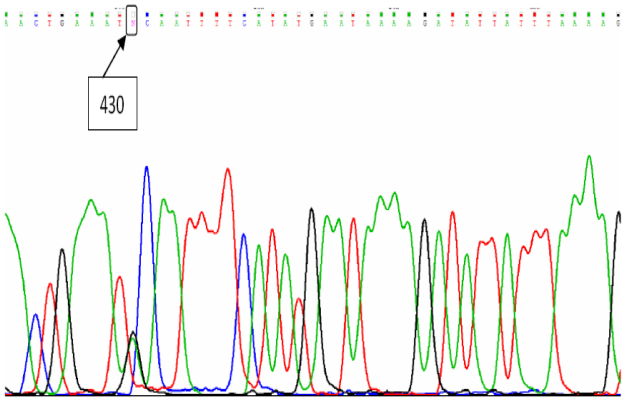

Figure 1 AAATACAATT→AAATGCAATT

SNPs were found, of which the first SNP was observed in position 430 in several sheep. In this position, adenine has been replaced by guanine. The change is a point mutation of the transition type, which showed the substitution. This SNP is located at OCT1, which has no effect on transcription recognition of heterozygosity in this position is shown in action or natural polymorphism (Figure 1).

In position 450 in all sheep, adenine was inserted to the E7-Box of the myostatin gene promoter. All samples for this mutation were homozygous. This SNP may act as a functional mutation. The transcription factor binding to this region was Cdx, which is disrupted by this SNP and has shifted to change the frame (Figure 2).

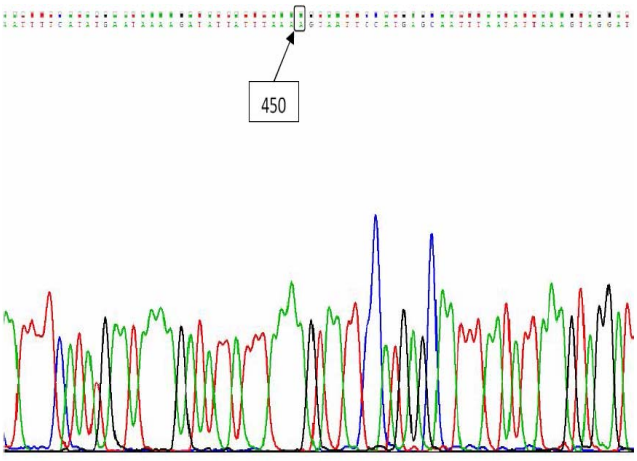

Figure 2 TAAAGATAT→TAAAAGATAT

Table 1 Primer Sequence for sequencing the promoter region of the ovis aries MSTN gene

Primer sequence	Fragment size (bp)	Annealing temp (˚C)	Gen Bank accession no
F: 5-GTACCAGCACAGTAGTGAGAAGC-3 R: 5-AGCCAAACGTTAATGCCTGC-3	1034	63	AY.918121

The last SNP of MSTN gene promoter is homozygosis that, in all samples was observed on position 540. Studies showed that this mutation has changed the recognized sequence by MZF1 factor. In this position the mutation sequence is similar to the sequence identified by the STAT protein. Changing protein caused a disruption to the transcription factor (Figure 3).

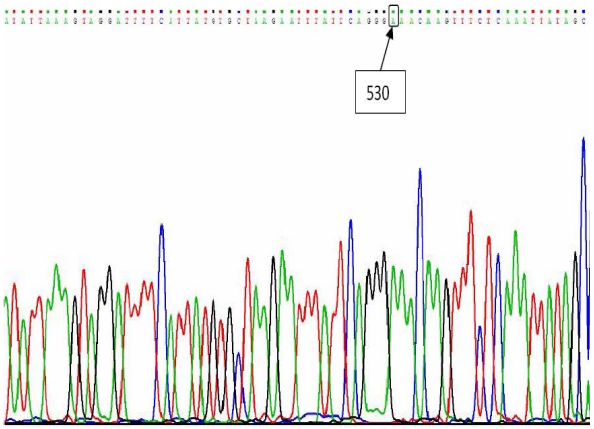

Figure 3 GGAACAAGTT→GAAACAAGTT

The Myostatin gene is a negative regulator of skeletal muscle growth. Muscle growth is a positive factor in cases of muscle weakness especially in muscular dystrophy. Muscular dystrophy is a disease that causes damage to muscle tissue, for instance Duchenne muscular dystrophy. In the various studies that have been published to determine gene mutation, different methods such as protein truanted test (PTT), single strand conformation polymorphism (SSCP), denaturing, hetero duplex analysis, gradient electrophoresis (DGGE) and direct sequencing have been used. The length of the myostatin gene promoter is 1.2 kb (Rong *et al.* 2007). Studies have been done on the majority of myostatin gene regions of exon and intron and gene promoter GDF8 research and possible mutations in this region have not been registered and no published data from other studies on this part of the myostatin gene are available, neither is information on boxes and sequence. As a partly regulatory gene, it is sensitive. The first step of gene expression in eukaryotes and prokaryotes is attributed to the promoter. Investigations of polymorphism in prokaryotes is sensible regulate gene expression in eukaryotes has been takes largely at the stage of transcription. The gene promoter is involved in the second stage; the change takes place and increases access to promoter transcription factors. Therefore, it was decided that a promoter review be conducted through systematic and compared sequencing. The promoter has been sensitive to mutations in its involvement of binding sites to protein (consensus sequences). Mutation changes the nucleotides in interactions of protein-DNA. Mutation has an affect on protected sequences of gene expression. Therefore, these mutations have the ability to disable the promoter or regulatory sequences and predict results based on gene expression. In this study, the MSTN promoter was sequenced. The advantage of this method compared to the other methods such as PCR-RFLP is that in sequencing, all possible changes are made clear, but PCR-RFLP techniques only show the genotype. Sequences showed changes in different parts of the samples. Eight samples out of 30 were heterozygous, in which position 430 showed that A was replaced by G in the OCT-1 binding site. This point mutation was a meaningless mutation because it does not change transcription. Position 450 in all samples showed insertion mutation, which was located in the protein-DNA bind and located in the myoblast consensus sequence, which damaged transcription and changed the frame performance of the myostatin gene. Insertion mutation is located in the active region of the promoter. Considering the effect of mutations in the myostatin gene, animals of phenotypic mutations must demonstrate a difference with normal animals. Position 530 exists in all samples; it is therefore homozygous. Mutations in this section are a point mutation type transition, which cause altered TF binding. Phenotype of these sheep showed differences of appearance from the Kordi sheep as well as muscle hypertrophy whereas Arabic sheep did not demonstrate hypertrophy. These results show that each of these mutations may change in terms of frame shift causing damage and preventing transcription or reduced gene expression. The promoter is a sensitive part and the smallest changes in active division of regulatory regions cause disruption in the transcription complex. Polymorphism in this study for Arabic sheep were homozygous for the myostatin gene, no change in volume and muscle mass was consistent with the results of Corsi *et al.* (2002) in cow but was inconsistent the results with Grobet *et al.* (1997) in cattle. This study is the first research on the MSTN gene promoter in Iranian sheep.The evident no change in muscle mass could also have derived from other causes:

1. The effect of other genes on muscle and meat. Considering that a quantitative trait is caused by many genes with small effects and is controlled by interactions between them.
2. Managerial factors, especially those of nutrition and environmental conditions probably contributed to the prevention of muscle phenotypes because this phenotype is affected by sex and feed.

CONCLUSION

This study is the first research on SNP in MSTN gene of Iranian sheep, which has led to the identification of SNPs promoter. Further analysis is required to confirm the results

of this research; investigation of the association of SNP with carcass characteristics is particularly recommended.

REFERENCES

Arthur P., Makarchain M. and Price M. (1998). Incidence of dystocia and prental calf mortality from reciprocal crossing of double-muscled and normal cattle. *J. Canadian Vet*. **29,** 163-167.

Belling R.H., Liberles D.A., Laschi S.P., Brien P. and Tay G. (2005). Myostatin and its implications on animal breeding. *J. Anim. Gen*. **36,** 1-6.

Boom R., Sol C.J.A., Salimans M.M.M., Jansen C.I. and Wherteim Van Dillen P.M.E. (1989). Rapid and simple method for purification of nucleic acid. *J. Clin. Microbiol*. **28,** 495-503.

Casas E., Shackerford S., Keele J., Stone R.T., Kappes S.M. and Koohmareie M. (2000). Quantitative trait loci affecting growth and carcass composition of cattle segregating alternate from of myostatin. *J. Anim. Sci*. **78,** 560-569.

Cieslak D., Blicharski T., Kapelanski W. and Pierzchala M. (2003). Investigation of polymorphism in the porcine GDF8 gene. *J. Anim. Sci*. **48,** 69-75.

Corsi A., Ferrucci L., Gozzini A., Tanini A. and Brandi M. (2002). Myostatin polymorphisms and age-related sarcopenia in the Italian population, *J. Am. Geriatr. Soc*. **50(8),** 1463-1467.

Fahrankrug S.C., Casas E., Keel J.W. and Smith T.D. (1999). Direct genotyping of the double-muscling locus in Piedmotese and Belgain blue cattle by fluorescent PCR. *J. Anim. Sci*. **77,** 2028-2030.

Ferrel R.E., Conte V., Lawerece E., Roth S., Hagberg J. and Hurely B. (1999). Frequent sequence variation in the human myostatin gene as a marker for analaysis of muscle-related phenotypes. *J. Genom*. **62(2),** 203-207.

Grobet L., Poncelet D., Pirottin D., Brouwers B., Requet J., Schoeberlein A. and Dunner S. (1998). A deletion in the bovine myostatine gene cause the double muscled phenotype in cattle. *J. Natu. Genet*. **17,** 71-73.

Kambadur R., Sharma M., Smith T. and Bass J. (1997). Mutation in myostatin in double-muscled Belgian Blue and Piedmontese cattle. *J. Gen. Res*. **7,** 910-915.

Karim L., Coppieters W., Grobet L., Valentini A. and Georges M. (2000). Convenient genotyping of six myostatin mutations causing double- muscling in cattle using a multiplex oligo nucleotide ligation assay. *J. Anim. Gen*. **31,** 396- 399.

Mcpherron A.C. and lee S.J. (1998). Double muscling in cattle due to mutation in the myostatin gene. *Pioc. Natl. Acad. Sci. USA*. **94,** 12457-12461.

Rong D., Xiaorong A., Yong C.H. and Jian Q. (2007). Functional analaysis of the myostatin gene promoter in sheep. *J. Life Sci*. **50,** 648-654.

Sofi B., Shojaeian K., Mohammad abadi M., Baghi Zadeh A. and Ferasati S. (2010). Myostatin gene polymorphism in Sanjabi sheep breed using molecular marker. *J. Anim. Sci. Rea*. **19,** 79-88.