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Molecular Study on the Exon 2 Region of the Ovis Bone Morphology Protein 15 (BMP-15) Gene in Iranian Bluchi Sheep Breed by PCR-SSCP Technique

Research Article

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ABSTRACT

Litter size is one of the most important economical traits in sheep breeding industry. In addition to minor genes, litter size is under the influence of major genes. Bone morphogenetic protein 15 (BMP-15), a member of the transforming growth factor beta (TGF-ß) superfamily, which is specifically expressed in oocytes, plays a dramatic role in sheep prolificacy. Reported mutations in this gene cause increased ovulation rate and infertility in a dosage-sensitive manner. Six different point mutations have been indicated in the BMP-15 gene of sheep, each having a major effect on prolificacy. The aim of the current study was to investigate the Lacaune ($FecX^L$) mutation in the prolificacy of the Iranian Baluchi sheep breed. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) coated tubes from jugular vein and genomic DNA was extracted from whole blood samples. Single nucleotide polymorphism of $FecX^L$ loci in BMP-15 gene were determined using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) technique. No evidence of mutation in $FecX^L$ was observed, all of which were monomorphic for exon 2 BMP-15 gene.

KEY WORDS BMP-15, $FecX^L$, gene, litter size, polymorphism, TGF-ß.

INTRODUCTION

There are more than fifty million head of sheep of more than twenty breeds and sub-breeds in Iran. Baluchi sheep is the most common native breed of sheep in Iran, comprising 30% of the sheep population. This breed is native to the eastern part of the country and widespread in the south parts of Khorasan, Sistan and Balouchestan, Yazd and Kerman provinces, which are mainly arid zones of the country. The breed is fat-tailed, having white fleece with black markings on the head and legs. This breed is primarily used for meat production, but also useful for wool production for the textile industry (Gholibeikifard *et al.* 2013). Prolificacy is measured by the ewe's ability to produce multiple lambs e.g. twins and triplets, through high ovulation rate and high survival rate of embryos.

There are several factors affecting ovulation rate of ewes which include genetics, stress, health, pasture type and quality, and ewe age and weight (Kareta *et al.* 2006). Genetic studies have indicated that the litter size and ovulation rate can be genetically determined by the action of single genes with a major effect in some European sheep breeds. The TGF-ß superfamily contains over 35 members, many of which have been shown to have a vital role in fertility

regulation (Knight and Glister, 2006). The TGF-ß members are multifunctional proteins which act through specific receptors to regulate growth and differentiation in many cell types, including those within the ovary (Elvin *et al.* 2000). They also play key roles in fertility and also during the embryogenesis period in mammals.

So far, three prolificacy loci related oocyte derived members of the TGF-ß superfamily have been discovered in sheep, namely bone morphogenetic protein receptor type 1B (BMPR1B; or activin-like kinase 6, ALK6), known as FecB (Booroola) on chromosome 6 (Souza *et al*. 2001) corresponding to the human chromosome 4q22-23 (Montgomery *et al*. 1993); growth differentiation factor 9 (GDF9), known as FecG on chromosome 5 (Hanrahan *et al.* 2004); and bone morphogenetic protein 15 (BMP-15; also known as GDF-9B) known as FecX on chromosome X (Galloway *et al*. 2000; Hanrahan *et al*. 2004). BMP-15, which is specifically expressed in oocytes, is essential for sheep prolificacy. Reported mutations in this gene cause increased ovulation rate and infertility in a dosage-sensitive manner. Critical roles of BMP-15 in female fertility have also been demonstrated in women (Di Pasquale *et al.* 2004). Lately, the roles of BMPs in embryonic development and cellular functions in postnatal and adult animals have been extensively studied (Chena *et al.* 2004).

In sheep, six different point mutations $FecX^{I}$ (Inverdale); $FecX^{H}$ (Hanna) (Galloway *et al.* 2000); $FecX^{L}$ (Lacaune) (Bodin *et al.* 2007); $FecX^{G}$ (Galway); $FecX^{B}$ (Belclare) (Hanrahan *et al.* 2004) and a 17 bp deletion of the functional gene ($FecX^{R}$) in Rasa Aragonesa sheep breed (Monteagudo *et al.* 2009) have been identified in the BMP-15 gene, each having a major effect on prolificacy. Ewes with two inactive copies of the BMP-15 gene (homozygous animals) are sterile (Galloway *et al.* 2000; Hanrahan *et al.* 2004) and have a similar ovarian phenotype, although those who have a single inactive BMP-15 gene (heterozygous animals) are fertile and have an increased ovulation rate and a higher incidence of twin or triplet births (Davis *et al.* 1991; Galloway *et al.* 2000; Hanrahan *et al.* 2004). The aim of the present study was to molecular study on the exon 2 region of the ovis BMP-15 gene in Baluchi sheep breed by PCR-SSCP technique.

MATERIALS AND METHODS

Sample collection and DNA isolation

Two hundred Iranian Baluchi sheep from animal breeding station of Abbasabad in northeast of Khorasan province in Iran were used in this study. The animals were used in accordance with the guidelines for the use and care of experimental animals and approved by the animal ethical committee of Tehran university, Iran, Islamic Republic of Iran. Blood samples (5 mL) were collected in EDTA vacutainer colleting tubes and transported to the laboratory in cool condition. Genomic DNA was extracted from whole blood samples by using a commercially available kit (Diatom of Isogene Lab. Ltd Co., Moscow, Russia). After measuring the DNA concentration and its purity (quality and quantity) by spectrophotometer and agarose gel electrophoresis, DNA was diluted to a final concentration of 50 ng/µL in sterile distilled water and kept at -20 ˚C for future use.

Primers for polymerase chain reaction (PCR) amplification

Specific primers (Table 1) were designed to amplify one region of BMP-15 gene (Exon 2) which corresponded to the Gen Bank accession number AH009593.1. The primers were designed using the primer premier 5.0 software. This software produced many sets of primers, which were all entered into the basic local alignment search tool (BLAST) to search for homology between the sequences and other genes in GenBank. The primer showing homology with gene other than the mentioned one was discarded.

PCR amplification

PCR amplification was carried out in 0.5 mL PCR tubes (Diatom of Isogene Lab. Ltd Co., Moscow, Russia). Each tube contained 1.5 units of *Taq* DNA polymerase, 10 m*M* of Tris-HCl (pH 9), 50 m*M* of KCl, 1.5 m*M* of $MgCl_2$ and 200 m*M* of each dNTP. 1 µL of each primer (50 ng/µL), 22 µL of water, 1 µL of genomic DNA (50 ng/µL) and two drops of mineral oil were added to this mixture. The amplification conditions for primers of the BMP-15 gene were as follows: denaturation at 95 ˚C for 5 min; followed by 35 cycles of denaturation at 94 ˚C for 30 s, annealing at (mostly around 53-56 ˚C) for 30 s and extension at 72 ˚C for 45 s; with a final extension at 72 ˚C for 10 min, on a thermocycler (Biometra T-Personal Ver: 1.11). Amplification was verified by electrophoresis on 2% (w/v) agarose gel in 1 X TBE buffer (2 m*M* of EDTA, 90 m*M* of tris-borate, pH 8.3), using a 100 bp ladder as a molecular weight marker for confirmation of the length of the PCR products. Gels were stained with ethidium bromide (1 µg/mL).

SSCP conditions

PCR products were resolved by SSCP analysis. Several methods including various steps, were tested for each fragment in order to optimize the methodology: amount of PCR product (4 µL to 13 µL), dilution in denaturing solution (25% to 70%), denaturing solution (A: 95% of formamide, 10 m*M* NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue; B: same as A, plus 20 m*M* of EDTA), acrylamide concentration (6% to 15%), voltage (100 to 400 V), running time (4 to 14 h) and running temperature (4, 7, 10 and 15

˚C). Each PCR reaction was diluted in denaturing solution, denatured at 95 ˚C for 5 min, cooled on ice and resolved by polyacrylamide gel electrophoresis. The electrophoresis was carried out in a vertical unit (160×140×1.0 mm spacers), in 1X TBE buffer. The gels were stained with silver nitrate. The conditions of SSCP analysis in the current study are described in Table 2.

DNA sequencing

Twenty randomly selected samples of PCR products from homozygote and heterozygote animals were used for sequencing. Primers, dNTP, buffer ingredients and nonspecific products were isolated, and then sequencing was done using a 3730 sequencer (Applied Biosystems 3730×l DNA Analyzer, Bioneer Company, South Korea). The sequenced fragments were aligned next to each other and single nucleotide polymorphism (SNP) was identified by sequence traces in contrast to original sequences in a livestock genomics database: (http://www.livestockgenomics.csiro.au/blast/).

The generated sequence data was further analyzed by using laser gene software (Burland, 2000). Multiple sequence alignment was performed using MegAlign program of laser gene software.

RESULTS AND DISCUSSION

The fragment of the BMP-15 gene (310 bp) was successfully amplified from the DNA of each sample (200 samples) used in the present study. Figure 1 shows agarose gel electrophoresis of the PCR amplified 310 bp fragment from exon 2 of ovine BMP-15 gene of Baluchi breed.

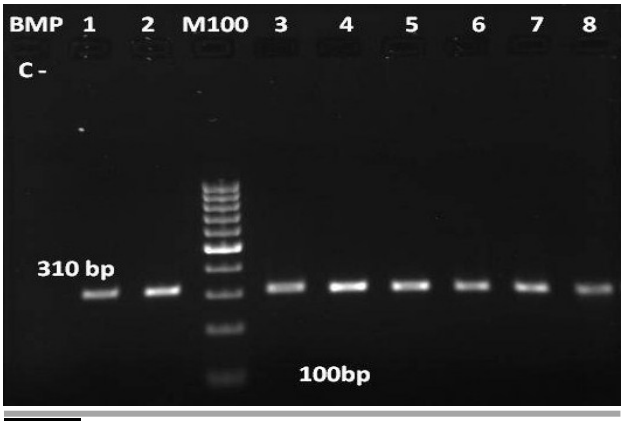

Figure 1 PCR products of BMP-15 gene, in Iranian Baluchi sheep breed analyzed by electrophoresis in a 2% agarose gel with ethidium bromide staining

After optimization of the parameters which affect the detection of SSCP's, the PCR products (n=200) animals were analyzed under the conditions described in Table 2. In the PCR-SSCP analysis of BMP-15 gene (exon 2) revealed one allele was identified and designated as A on the basis of electrophoretic mobility in the gel. We obtained one conformational pattern (genotype), AA (Figure 2). The amplification was not possible in ten samples (5%).

Allele and genotype frequencies

The allele and genotype frequencies of this gene in Iranian Baluchi sheep breed are shown in Table 3. For BMP-15 gene, the genotype frequencies were 100 and 0.0% for AA and AB, respectively; the allele frequencies for the A and B were 1.0 and 0.0, respectively.

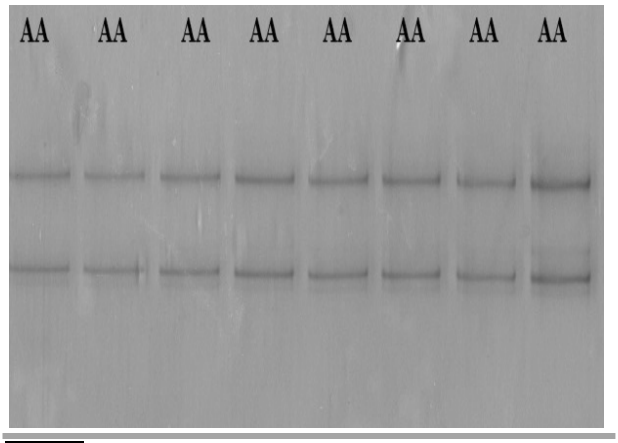

Figure 2 The SSCP analysis of the 310 bp fragment for BMP15 gene polymorphism in Iranian Baluchi sheep breed

Sequencing results

The results obtained after SSCP analysis were confirmed by sequencing twenty randomly selected DNA samples for each primer pair, in both forward and reverse directions for BMP-15. In the present study, DNA sequencing analysis showed that the sequences of PCR products corresponded to the sequences (Figure 3) in the GenBank. In SSCP analysis and sequencing nucleotide variation for the BMP-15 gene was not detected. In this case, more studies are needed in order to better understanding the importance of this gene, which has never been described in other breeds.

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Query  62   CAGCAGCTGGGCTGGGATCACTGGATCATTGCTCCCCATCTCTATACCCCAAACTACTGT 121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  904  CAGCAGCTGGGCTGGGATCACTGGATCATTGCTCCCCATCTCTATACCCCAAACTACTGT 963
```

Figure 3 Nucleotide sequence comparison of the PCR products of primers AH009593.1

BLAST results of the nt sequence of genotype AA of BMP-15 gene in Iranian Baluchi sheep breed with previously published nt sequence in Gen Bank. (Query, our samples) compared with sheep sequence registered in the Gene Bank (Subject)

Mutations at five different points in exon 2 of BMP-15 gene are associated with prolificacy in some breeds of sheep (Montgomery *et al.* 2001). Mutations in fecundity genes like BMP-15 have important economic values in sheep breeding and probably ruminant reproduction (Galloway *et al.* 2000; Hanrahan *et al.* 2004; McNatty *et al.* 2005).

Table 1 Primer sequences, amplified region and fragment size for PCR amplification in SSCP analysis of the BMP-15 gene in Iranian Baluchi sheep breed

Candidate gene	Primer sequences (5′ to 3′)	Amplifiedregion	Fragment size (bp)	Gen Bank accession no.
BMP-15	Fw-GCAGGCAGTATTGCATCGGAAG Rw-CCTCAATCAGAAGGATGCTAATGG	Exon 2	310	AH009593.1

BMP-15: bone morphogenetic protein 15.

Table 2 Conditions of electrophoresis in SSCP analysis of PCR products of BMP-15 gene in Iranian Baluchi sheep breed

Gene	Acryl amide	DNA	Denaturing solution	Temperature	Duration	Voltage
BMP-15 (Exon 2)	10%	8 µL	12 µL	5 °C	7 h	300 V

BMP-15: bone morphogenetic protein 15.

Table 3 Genotype and allele frequencies for BMP-15 gene in Iranian Baluchi sheep population (n=190)

Candidate gene	Genotype frequencies		Allele frequencies	
	AA	AB	A	B
BMP-15 (Exone 2)	190 (100)[1]	0 (0.0)	1.0	0.0

[1]Figures within parentheses are the percentage.
BMP-15: bone morphogenetic protein 15.

Naturally occurring heterozygous mutations in BMP-15 in sheep increase the ovulation rate and prolificacy, whereas homozygous mutations yield infertile animals (Galloway *et al.* 2000; Hanrahan *et al.* 2004).

The results of our study detected no polymorphism in $FecX^L$ gene (having a major effect on litter size) in Baluchi sheep and all of them were monomorphic for this locus. These results are in agreement with reports of Romanov, Finn, East Friesian, Teeswater, Blueface Leicester, D'Man, Chios, Mountain Sheep, German Whiteheaded Mutton, Lleyn, Loa, Galician, Barbados Blackbelly sheep (Davis *et al.* 2006), and Iranian goats (Deldar-Tajangookeh *et al.* 2009). In the present study, tests were carried out only for the Lacaune $FecX^L$ mutation and we did not find any genetic variations within the BMP-15 gene by PCR-SSCP among 200 individual Baluchi sheep. Zare *et al.* (2007) also detected no mutations in two points of BMP-15 gene ($FecX^G$ and $FecX^L$) from 240 blood samples of Shal ewes by using of PCR-RFLP and PCR-SSCP techniques. Nejati-Javaremi *et al.* (2007) using PCR-SCCP techniques investigated the polymorphism in $FecX^L$ gene associated with twinning in Iranian Lori-Bakhtiari sheep but found no differences in the band pattern of denatured PCR products.

CONCLUSION

The results of the present study show that there is no genetic polymorphism of $FecX^L$ loci in BMP-15 gene in Iranian Baluchi sheep. Further investigation should be directed at other loci of BMP-15 gene or other genes, using larger sample sizes.

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REFERENCES

Bodin L., Di Pasquale E., Fabre S., Bontoux M., Monget P., Persani L. and Mulsant P. (2007). A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. *Endocrinology.* **148,** 393-400.

Burland T.G. (2000). DNA STAR's Lasergene sequence analysis software. *Methods Mol. Biol.* **132,** 71-91.

Chena D., Ming Z. and Gregory R. (2004). Bone morphogenetic proteins. *Grow. Fact.* **22,** 233-241.

Davis G.H., McEwan J.C., Fennessy P.F., Dodds K.G. and Farquhar P.A. (1991). Evidence for the presence of a major gene influencing ovulation rate on the X-chromosome of sheep. *Biol. Reprod.* **44,** 620-624.

Davis G.H., Balakrishnan L., Ross I.K., Wilson T., Galloway S.M., Lumsden B.M., Hanrahan J.P., Mullen M., Mao X.Z., Wang G.L., Zhao Z.S., Zeng Y.Q., Robinson J.J., Mavrogenis A.P., Papachristoforou C., Peter C., Baumung R., Cardyn P., Boujenane I., Cockett N.E., Eythorsdottir E., Arranz J.J. and Notter D.R. (2006). Investigation of the Booroola (FecB) and Inverdale (FecX (I)) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. *Anim. Reprod. Sci.* **92,** 87-96.

Deldar-Tajangookeh A., Zare Shahneh A., Zamiri M.J., Daliri M., Kohram H. and Nejati-Javaremi A. (2009). Study of BMP-15 gene polymorphism in Iranian goats. *African J. Biotechnol.* **8,** 2929-2932.

Di Pasquale E., Beck-Peccoz P. and Persani L. (2004). Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *Am. J. Human Gen.* **75,** 106-111.

Elvin J.A., Yan C. and Malsuk M.M. (2000). Oocytes-expressed TGF-beta superfamily members in female fertility. *Mol. Cell. Endocrinol.* **159,** 1-5.

Galloway S.M., McNatty K.P., Cambridge L.M., Laitinen M.P.E., Juengel J.L., Jokiranta T.S., McLaren R.J., Luiro K., Dodds K.G. and Montgomery G.W. (2000). Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat. Genet.* **25,** 279-283.

Gholibeikifard A., Aminafshar M. and Hosseinpour Mashhadi M. (2013). Polymorphism of IGF-I and ADRB3 genes and their association with growth traits in the Iranian Baluchi sheep. *J. Agric. Sci. Technol.* **15,** 1153-1162.

Hanrahan J.P., Gergan S.M., Mulsant P., Mullen M., Davis G.H., Powell R. and Galloway S.M. (2004). Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in cambridge and belclare sheep (*Ovis aries*). *Biol. Reprod.* **70,** 9000-9009.

Kareta W., Korman K. and Cegla M. (2006). Ovulation level and prolificacy in ewes depending on their age, birth type and percentage of prolific genotype. *Reprod. Biol.* **6,** 73-78.

Knight P.G. and Glister C. (2006). Focus on TGF-ß signalling: TGF-ß superfamily members and ovarian follicle development. *Reproduction.* **132,** 191-206.

McNatty K.P., Smith P., Moore L.G., Reader K., Lun S., Hanrahan J.P., Groome N.P., Laitinen M., Ritvos O. and Juengel J.L. (2005). Oocyte expressed genes affecting ovulation rate. *Mol. Cell. Endocrinology.* **234,** 57-66.

Monteagudo L.V., Ponz R., Tejedor M.T., Laviña A. and Sierra I. (2009). A 17bp deletion in the bone morphogenetic protein 15 (BMP15) gene is associated to increased prolificacy in the Rasa Aragonesa sheep breed. *Anim. Reprod. Sci.* **110,** 139-146.

Montgomery G.W., Crawford A.M., Penty J.M., Dodds K.G., Ede A.J., Henry H.M., Pierson C.A., Lord E.A., Galloway S.M., Schmack A.E., Sise J.A., Swarbrick P.A., Hanrahan V., Buchanan F.C. and Hill D.F. (1993). The ovine Booroola fecundity gene (FecB) is linked to markers from a region of human chromosome 4q. *Nat. Genet.* **4,** 410-414.

Montgomery G.W., Galloway S.M., George H., Davis G.H. and McNatty K.P. (2001). Genes controlling ovulation rate in sheep. *Reproduction.* **121,** 843-852.

Nejati-Javaremi A., Rahimi G. and A.A. (2007). Detection of polymorphism in $FecX^{L}$ gene associated with twinning in Lori-Bakhtiari sheep breed using PCR-SSCP. Pp. 520 in Proc. 5th Natio. Biotechnol. Cong. Iran. Tehran, Iran.

Souza C.J.L., MacDougall C., Campbell B.K., McNeilly A.S. and Baird D.T. (2001). The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. *J. Endocrinol.* **169,** 1-6.

Zare Y., Nejati-Javaremi A. and Rahimi G. 2007. Detection of polymorphisms in two point of gene associated with Twinning (BMP-15) in Shal sheep. Pp. 483 in Proc. 5th Natio. Biotechnol. Cong. Iran. Tehran, Iran.