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Ovine relaxin family peptide receptor 2 (RXFP2) gene polymorphism – no association with cryptorchidism

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ABSTRACT

The polymorphism in ovine relaxin family peptide receptor 2 gene and the relevance of earlier established human and mice cryptorchidism associated SNP's in Mandya and Hassan Sheep was studied. Genomic DNA was extracted from 60 cryptorchid and 80 normal unrelated sheep. Two sets of primers were designed to amplify exon 8 and exon 12–13 regions of ovine RXFP2 gene. SSCP revealed no polymorphism at exon 8, exon 12 and exon 13 of ovine RXFP2 indicating absence of T222P in exon 8 and D294G in exon 12 as reported in human and mice cryptorchids respectively. A novel SNP (KF527573.1, 171T>A) in intron12 of ovine RXFP2 was observed. The sheep population studied was in Hardy Weinberg equilibrium for the genotypes of the SNP. The distribution of genotypes was significantly different for Hassan and Mandya sheep breeds. However, the SNP in both the breeds studied was not associated with the cryptorchid phenotype.

Key words: Cryptorchidism, Mandya, Ovine, Polymorphism, RXFP2

Cryptorchidism is the failure of one or both testes to be positioned in the scrotum at the time normal for a species. It is the most common genetic defect of the male genital system. In animal breeding, the occurrence of cryptorchidism leads to economic loss and decreased selection potential of male breeding stock. The prevalence of cryptorchidism is common in humans, pigs, horses and companion animals (2–12%) and are said to be rare in general populations of cattle, sheep and goat (Amann and Veeramachaneni 2007). But extremely high frequencies of cryptorchidism had been reported in certain breeds of sheep (Polled Merino and Karagouniko). Among Indian sheep breeds, Mandya breed is having high prevalence of cryptorchidism and the major cause for decline in its population (Bhatia and Arora, 2005).

Etiology for cryptorchidism involves genetic, epigenetic, environmental factors and their interactions. The studies have elucidated that the major regulators of testicular descent are the Leydig cell–derived hormones insulin-like factor 3 (INSL3) and testosterone and their receptors involved, respectively, in the transabdominal and inguinoscrotal phase of testicular descent.

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The INSL3 protein is a testicular hormone produced by leydig cells, belonging to relaxin family of protein. The INSL3 gene structure comprising of two exons and one intron is highly conserved among mammals, including sheep. Several mutations of human INSL3 are strongly associated with cryptorchidism. Williams *et al.* (2007) reported association of Ovine INSL3 2489C>T SNP in intron 1 with cryptorchidism.

Relaxin family peptide receptor 2 (RXFP2) is the only receptor for INLS3, hence one of the potential candidate gene for cryptorchdism. RXFP2 also called as 'Great' (Overbeek *et al.* 2001), LGR8 (Kumagai *et al.* 2002) belong to the family of G-protein-coupled receptor (GPCR), as three glycoprotein hormone receptors (FSHR, LHR and TSHR). Expression of RXFP2 is seen in the testis, ovary, brain and skeletal muscles, with the highest level of expression in the gubernaculum. RXFP2 is said to be also involved in male gonad development, negative regulation of apoptosis, negative regulation of cell proliferation, oocyte maturation and positive regulation of cAMP biosynthetic process (Feng *et al.* 2007). Further, Zhangyuan *et al.* (2018) confirmed the role of ovine RXFP2 in development of horn pattern as response to semi feralization.

Decreased or defective expression of the receptor is involved in intra abdominal cryptorchidism in mice and human (Overbeek *et al.* 2001, Bathgate *et al.* 2006). RXFP2 gene is relatively conserved across broad spectrum of species and has 18 exons. Several mutations had been reported in the RXFP2 gene of which a SNP in exon 8, corresponding to T222P mutation of RXFP2 in human (Nuti

et al. 2007) and a SNP in exon 12 corresponding to D294G mutation of RXFP2 in mouse (Harris *et al.* 2010) were reported to be involved in cryptorchidism. Hence, in the present study, polymorphism of exon 8 and exon12–13 of ovine RXFP2 gene and their probable association with cryptorchidism were studied.

MATERIALS AND METHODS

The blood samples were collected for isolation of genomic DNA and further genetic studies from a total of 140 unrelated sheep (60 cryptorchid and 80 normal males) belonging to Mandya and Hassan breeds of sheep. DNA extraction was carried out adopting high salt method as described by Miller *et al.* (1988). Quality, purity and concentration of genomic DNA were confirmed by agarose gel electrophoresis and UV-photometric methods. Two sets of primers were designed based on the partial sequence of Ovine RXFP2 obtained from BLAST of the bovine homologous (ENSBTAT00000020135) from Ensemble data base on to the ovine whole genome database (Oar_v3.1) published by the International Sheep Genomics Consortium (ISGC). Primer select program of CLC BIO software was used for designing primers. The details of the oligonucleotide sequence, their Tm values and product size are presented in Table 1.

Polymerase chain reaction was carried out in final reaction volume of 25 µl, containing 100 ng of template, 5 pmol of each primer, 200 M of each dNTP, 2.5 µl of 10× buffer with 1.5 mM $MgCl_2$ and one unit of *Taq* DNA polymerase. The PCR cycle conditions include initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, respective annealing temperature for 30 sec and 72°C for 30 sec, with a final extension for 7 min at 72°C. The PCR products were visualized under 1.5% agarose gel and later subjected to single stranded confirmation polymorphism (SSCP) analysis. PCR products were diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA), heated at 95°C for 5 min followed by snap chilling for 5 min. 12.5% polyacrylamide gels were used for resolution. The gels were silver stained (Bassan *et al.* 1991), dried on cellophane sheet and scored manually for variants. The PCR products showing variants were purified and custom sequenced. Prediction of tolerant level of mutation on protein function was done using Sorting Tolerant from Intolerant (SIFT) algorithm. The Hardy Weinberg equilibrium of the population and association of SSCP variants with cryptorchidism was studied by Fishers exact test of SAS 9.2 software.

Table 1. Primers used for amplification of exon 8, exon 12–13 of ovine relaxin family receptor 2 gene

Amplicon		Sequence	Tm value	Annealing temp.	Product size
RXFP2-I	F	GTCAGAATTCTAG ATGACAATCCC	59.21	59°C	258 bp
	R	AAATATGCCAT GAGCCATGG	58.84		
RXFP2-II	F	TCACTGTCAC CAACAGGGAC	62.19	61°C	323 bp
	R	GAGTAAAGCAA CCCACTAACATC	59.82		

RESULTS AND DISCUSSION

PCR amplification of the RXFP2-I region resulted in a fragment of 258 bp covering exon 8 and part of intron 8 region of ovine RXFP2 gene. The ovine RXFP2 exon 8 was of 72 bp length and was highly conserved. Comparison of the translated exon 8 sequences from sheep and human revealed only one variant at 8th amino acid position i.e. threonine (T) in humans and serine (S) in sheep. Gorlov *et al.* (2002) reported that a substitution mutation threonine (T) to proline (P) at 8th amino acid position due to change of A to C at 23 nucleotide position in exon 8 of RXFP2 was involved in certain cases of human cryptorchidism. The SSCP study did not reveal any polymorphism of ovine RXFP2 exon 8, suggesting that this region is not involved in cryptorchidism in the sheep breeds studied. The sequence analysis revealed the presence of nucleotide 'G' in ovine RXFP2 resulting in serine at 8th amino acid position. Lack of conservation of the amino acid at 8th position of translated RXFP2 exon 8 across species questions the validity of the mutation as cause of cryptorchidism. Further, El Houate *et al*. (2008) had also reported this mutation to be not associated with human cryptorchidism.

PCR amplification of the RXFP2-II region resulted in a fragment of 323 bp covering exon 12, intron 12, exon 13 and part of intron 13 region of ovine RXFP2 gene. SSCP analysis showed variants. The analysis of ovine RXFP2 exon 12 and exon 13 sequences and their comparison with other species showed that the sequence length was 72 bp for both the exons and were conserved across species. The nucleotide sequence and translated amino acid sequences of exon 12 and exon 13 of sheep were compared with diverse mammalian species as mice and human. The translated sequences of RXFP2 exon 12 and 13 from sheep, human and mice and their comparison are presented in Fig. 1.

SNP in exon 12 corresponding to D294G mutation of RXFP2 in mouse was associated with cryptorchdism (Harris *et al.* 2010). In present study, Sorting Tolerant from Intolerant (SIFT) algorithm (http://sift.jcvi.org) predicted aspartic acid encoded by first codon of exon 12 as essential for normal functioning of the protein. The exon 12 sequence GAC (nucleotide 2–4) and the corresponding translated amino acid aspartic acid (D294) was found conserved in sheep sequence, whereas the human RXFP2 sequence revealed a silent mutation, i.e. GAT sequence with no alteration in amino acid (D294).

A novel SNP in intron12 of ovine RXFP2 was observed and the sequence submitted to GenBank with accession number KF527573.1 (171T>A). The sheep population studied was in Hardy Weinberg equilibrium for the genotypes of the SNP. The distribution of genotypes was

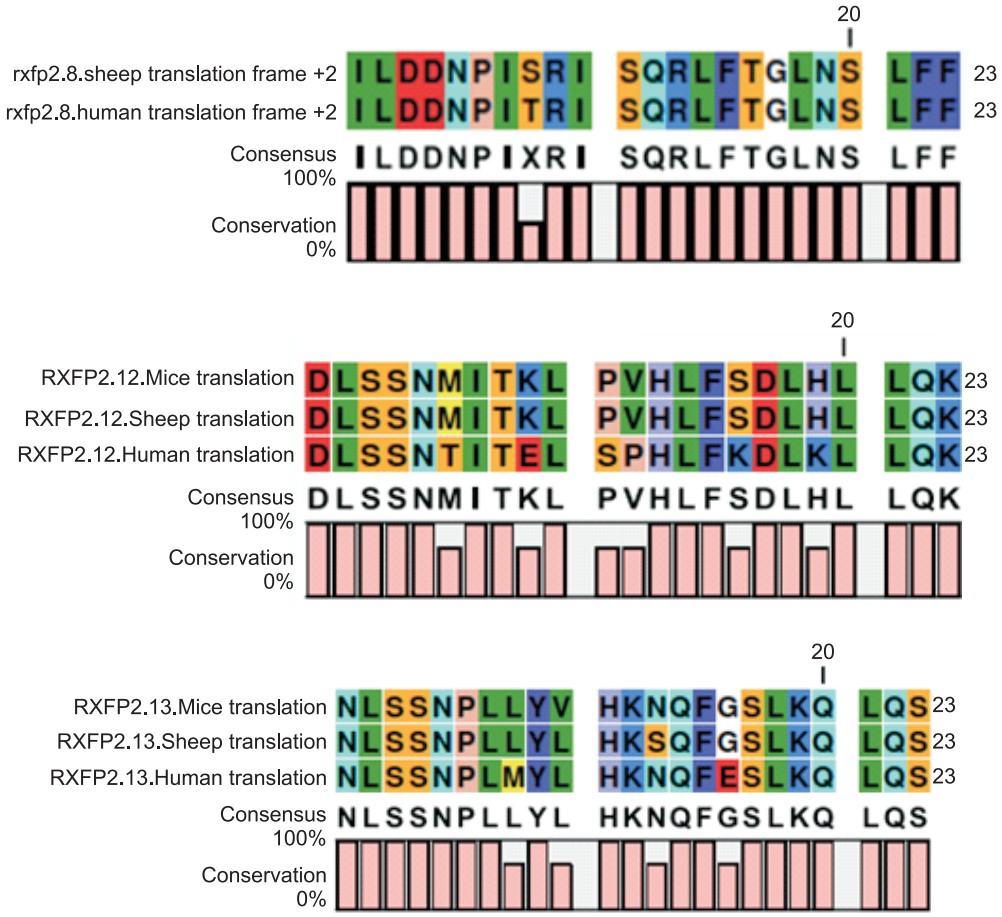

Fig. 1. Comparison of translated ovine RXFP2 with human and mice sequences.

Table 2. Ovine RXFP2 ((KF527573.1, 171T>A) SNP (Genotypes, their distribution among breeds and cryptorchid phenotype)

Breed	Phenotype	No of Sheep	Observed genotype frequency (No.)			P value for HWE
			TT	TA	AA	
Combined population	140	0.37 (52)	0.41 (57)	0.22 (31)	0.1030	
Mandya	Normal	47	0.47 (22)	0.38 (18)	0.15 (7)	0.5410
	Cryptorchid	35	0.49 (17)	0.40 (14)	0.11 (4)	0.8797
	Total	82	0.48 (39)	0.39 (32)	0.13 (11)	0.6018
P values for b/w phenotypes		0.8744	0.8758	0.6497		
Hassan	Normal	33	0.22 (7)	0.42 (14)	0.36 (12)	0.7575
	Cryptorchid	25	0.24 (6)	0.44 (11)	0.32 (8)	0.8333
	Total	58	0.23 (13)	0.43 (25)	0.34 (20)	0.6443
P values for b/w phenotypes		0.8010	0.9045	0.7293		
P values for b/w breeds		0.0029	0.6286	0.0041		

P>value of less than 0.05 indicates significant difference.

significantly different for Hassan and Mandya sheep breeds. Mandya breed had significantly higher frequency of TT genotype and significantly lower frequency of AA genotype than Hassan sheep breed. The relevance of this needs to be studied. However, the SNP in both the breeds studied was not associated with the cryptorchid phenotype. The genotype frequencies and their distributions are given in Table 2.

REFERENCES

Amann R P and Veeramachaneni D N R. 2007. Cryptorchidism in common eutherian mammals. *Reproduction* **133**: 541–61.

Bassam B J, Caetano-Anollés G and Gresshoff P M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anaytical Biochemistry* **196**(1): 80–83.

Bathgate R A, Hsueh A J, Ivell R, Sanborn B M, Sherwood O D and Summers R J. 2006. International Union of Pharmacology:

Recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacological Reviews* **58**: 7–31.

Bhatia S and Arora R 2005. Biodiversity and conservation of Indian sheep genetic resources an overview. *Asian-Australasian Journal of Animal Science* **18**: 1387–1402.

CLC BIO. 2011. CLC Genomic Workbench 6.5.1 Aarhus, Denmark. (http://www.clcbio.com).

El Houate B, Rouba H, Imken L, Sibai H, Chaûk A, Boulouiz R, Chadli E, Hassar M, Mcelreavey K and Barakat A. 2008. No association between T222P/LGR8 mutation and cryptorchidism in the Moroccan population. *Hormone Research* **70**: 236–39.

Feng S, Bogatcheva N V, Truong A, Korchin B, Bishop C E, Klonisch T, Agoulnik I U and Agoulnik A I. 2007. Developmental expression and gene regulation of insulin-like 3 receptor RXFP2 in mouse male reproductive organs. *Biology of Reproduction* **77**: 671–80.

Gorlov I P, Kamat A, Bogatcheva N V, Jones E, Lamb D J, Truong A, Bishop C E, Mcelreavey K and Agoulnik A I. 2002. Mutations of the GREAT gene cause cryptorchidism. *Human Molecular Genetics* **11**(19): 2309–18.

Harris R M, Finlayson C, Weiss J, Fisher L, Hurley L, Barrett T, Emge D, Bathgate R A, Agoulnik A I and Jameson J L. 2010. A missense mutation in LRR8 of RXFP2 is associated with cryptorchidism. *Mammalian Genome* **21**(9–10): 442–49.

Kumagai J, Hsu S Y, Matsumi H, Roh J S, Fu P, Wade J D, Bathgate R A and Hsueh A J. 2002. INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *Journal of Biological Chemistry* **277**: 31283–86.

Miller S A, Dykes D D and Polesky H F. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* **16**(3): 1215–18.

Nuti F, Marinari E, Erdei E, El-Hamshari M, Echevarria M G, Ars E, Balercia G, Merksz M, Giachini C and Schaeer K Z. 2008. The leucine-rich repeat-containing G protein-coupled receptor 8 gene T222P mutation does not cause cryptorchidism. *Journal of Clinical Endocrinology and Metabolism* **93**: 1072–76.

Overbeek P A, Gorlov I P, Sutherland R W, Houston J B, Harrison W R, Boettger-Tong H L, Bishop C E and Agoulnik A I. 2001. A transgenic insertion causing cryptorchidism in mice. *Genesis* **30**: 26–35.

SAS 9.2. 2009. Procedures Guide. SAS Institute Inc, Cary, NC, USA.

Zhangyuan P, Shengdi L, Qiuyue L, Zhen W, Zhengkui Z, Ran D, Benpeng M, Wenping H, Xiangyu W, Xiaoxiang H, Ze X, Dongkai W, Xiaoyun H, Liyun Y, Benmeng L, Ruichao W, Xiaoyu L, Xiaohan C, Xinlong D, Qing X, Hongcai S, Geng H, Jean Y, Cuicheng L, Yiqiang Z, Mei J, Yingjie Z, Shenjin L, Fukuan L, Guohui D, Mingxing C and Yixue L. 2018. Whole-genome sequences of 89 Chinese sheep suggest role of *RXFP2* in the development of unique horn phenotype as response to semi-feralization. *Giga Science* 7(4): giy019.