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# Ovine androgen receptor exon1 polymorphism - no association with cryptorchidism

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ABSTRACT

The exon1 of androgen receptor (AR) coding for N-terminal domain (NTD) has 3 regions of CAG and a GGC repetitive sequence coding for glutamine and glycine repeats respectively. The position and number of these trinucleotide repeats varies with the species and said to have evolutionary significance. In humans, shorter CAG repeats and longer GGC repeats are found to be associated with risk of cryptorchidism. The work was taken up to know the status of trinuclotide repeats and polymorphism in exon1 of ovine AR gene and its association with cryptorchidism. Partial sequences covering the repetitive regions of exon1 of ovine AR gene were PCR amplified from genetically unrelated 80 normal and 60 cryptorchid sheep. Single stranded conformation polymorphism and sequencing revealed no polymorphism at any of the 3 CAG sites and a GGC site of exon1 of ovine sequence. The number of trinucleotide repeats in ovine AR gene were 4, 7, 8 and 6 for CAG-I, CAG-II, CAG-III and GGC regions respectively. The 2 novel SNP 362 C>G and 603 C>T with the frequency of 0.03 and 0.27, respectively, in whole population was found unassociated with the cryptorchidism in sheep. Substitution mutation 362 C>G resulting in 121 Alanine>Glycine, was found to be non effective on protein function by SIFT algorithm.

**Key words**: Androgen receptor, Cryptorchidism, Polymorphism, Sheep, Trinuclotide

The androgen receptor (AR) is critical for the normal development and functioning of male reproductive system. The AR gene is conserved throughout mammalian evolution and consists of 8 exons. The transcriptional regulatory region of AR coded by the first exon contains CAG and GGC trinucleotide repeat regions. The position and polymorphism of these trinucleotide repeat regions varies with the species. Davis-Dao *et al*. (2012) reported association of trinucleotide repeat length in exon 1 with cryptorchidism. As, such studies from livestock species were scanty, the present work was taken up to know the polymorphism at exon1 of ovine androgen receptor gene and its association with cryptorchidism.

## MATERIALS AND METHODS

Blood samples were collected from 140 unrelated sheep (60 cryptorchid and 80 normal males) belonging to Mandya and Hassan breeds. Blood was collected from the jugular vein into EDTA containing vacutainer tubes and DNA extraction was performed from whole blood following a standard phenol-chloroform extraction method (Sambrook and Russell 2001). Three sets of the oligonucleotide primer pairs were designed based on the partial sequence of ovine androgen receptor exon 1 obtained from BLAST of bovine homologous androgen receptor exon1 sequence (ENSBTAT00000030067) from Ensemble on to whole genome database (Oar_v3.1). Primer select program of CLC-BIO software was used for designing primers. The details of the oligonucleotide sequence, their $T_m$ values and expected product size are presented in Table 1. Polymerase chain reaction was carried out in a final reaction volume of 25 µl, containing about 100 ng of genomic DNA, 5 pmole of each primer, 200 M of each dNTP, 2.5 L of 10x buffer with 1.5 mM MgCl and one unit of *Taq* DNA polymerase. Amplification was performed using a programmable thermal cycler with an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C – 30 sec, respective annealing temperature for 30 sec and 72°C – 30 sec, with a final extension for 7 min at 72°C. The PCR products were visualized following electrophoresis through a 1.5 to 2% ethidium bromide stained agarose gel. PCR products were subjected to single strand conformation polymorphism (SSCP) analysis. The third fragment of 429 bp was digested with *PstI* (as per suppliers specification) before PCR product was 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, snap chilled in ice for 3 min and resolved on 12% polyacrylamide gel. The electrophoresis was carried out in a Bengaluru genei vertical electrophoresis unit using 1x

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Table 1. Primer information and Gene bank acession number of ovine AR exon1 regions

| Amplicon | Forward primer | $T_m$ value | Product size | Gen bank accession No. |
|---|---|---|---|---|
| AR fragment1 | F: AAGTGATCCAGAACCCGCTC<br>R: AGCTGAGTCATCCTCATCCA | 62.4<br>60.53 | 323 | KC878300.1 |
| AR fragment2 | F: CTCCACCGATCTTAAAGACATCCT<br>R: AGATGCTCCAATGCCTCCAC | 62.25<br>61.49 | 246 | KC878301.1 |
| AR fragment3 | F: ACTCGCGACTACTACAACTTTCC<br>R: GGATAGGGCACTCTGCTCACC | 62.73<br>64.83 | 429 | KF554248.1 |

Table 2. Ovine AR exon 1SSCP pattern frequency and their distriibution

| Breed | Fragment- I | Cryptorchid | Normal | Total | Pr > ChiSq b/w phenotype |
|---|---|---|---|---|---|
| Mandya | SSCP- A | 0.8 (28/35) | 0.87 (41/47) | 0.84 (69/82) | 0.3784 |
| | SSCP- B | 0.2 (7/35) | 0.13 (6/47) | 0.16 (13/82) | |
| Hassan | SSCP- A | 0.6 (15/25) | 0.7 (23/33) | 0.66 (38/58) | 0.4428 |
| | SSCP- B | 0.4 (10/25) | 0.3 (10/33) | 0.34 (20/58) | |
| Total | SSCP- A | 0.72 (43/60) | 0.8 (64/80) | 0.76 (107/140) | 0.2522 |
| | SSCP- B | 0.28 (17/60) | 0.34 (26/80) | 0.24 (33/140) | |
| Pr > ChiSq b/w breeds | 0.0121* | | | | |
| Breed | Fragment- II | Cryptorchid | Normal | Total | Pr > ChiSq b/w phenotype |
| Mandya | SSCP-A | 1 (35/35) | 0.98 (46/47) | 0.99 (81/82) | 0.99 |
| | SSCP-B | 0 (0/35) | 0.02 (1/47) | 0.01 (1/82) | |
| Hassan | SSCP-A | 0.92 (23/25) | 0.88 (29/33) | 0.9 (52/58) | 0.6121 |
| | SSCP-B | 0.08 (2/25) | 0.12 (4/33) | 0.1 (6/58) | |
| Total | SSCP-A | 0.97 (58/60) | 0.94 (75/80) | 0.95 (133/140) | 0.4405 |
| | SSCP-B | 0.03 (2/60) | 0.06 (5/80) | 0.05 (7/140) | |

Pr > ChiSq b/w breeds 0.0412*. *Significant difference at a, 0.05%.

TBE buffer. Gels were silver-stained (Bassam *et al.* 1991), dried on cellophane sheet and scored manually for SSCP variants.

Primer pairs used for sequencing were the same as used for PCR-SSCP analysis. PCR products were purified by an enzymatic method using exonuclease I and antarctic phosphatase. Purified PCR products were custom sequenced. Prediction of tolerance level of mutation on protein function was done using SIFT algorithm (http://sift.jcvi.org). The association of SSCP variants with cryptorchidism was studied by Fishers exact test using proc freq of SAS 9.2 software.

## RESULTS AND DISCUSSION

The present study was taken up to identify the polymorphisms at exon1 of ovine androgen receptor gene and its role in cryptorchidism. The exon1 of the ovine AR gene was studied in 3 fragments corresponding to trinucleotide repeat regions. PCR-SSCP of the first fragment corresponding to CAG-I and CAG-II repetitive regions showed 2 types of banding pattern (Fig. 1.a). The sequencing revealed that these banding patterns are not due to variation in either CAG-I or CAG-II repeat number but due to a novel SNP i.e., guanine to cytosine at 362 bp of exon1. Only 2 types of banding patterns were observed as AR gene is a sex linked gene. The number of trinucleotide repeats at ovine CAG-I and CAG-II sites were 4 and 7, respectively, and were present 5 triplet codons apart. The substitution mutation (glycine with alanine at 121 amino acid) was in non conserved position. Sorting intolerant from tolerant (SIFT) algorithm predicted the substitution mutation as tolerant with score of 1.0 indicating no altered protein function thus nonsignificant in AR protein structure. The PCR SSCP banding pattern did not show any association with the cryptorchidism in the sheep population studied (Table 2). The sequence was the first ovine AR information to be submitted to public domain database (Gen Bank No. KC878300.1) and it revealed the sequence information of 51 bp which was present as a gap in Ora3.1 sheep genome database.

PCR-SSCP of the second fragment corresponding to CAG-III region revealed 2 types of banding patterns

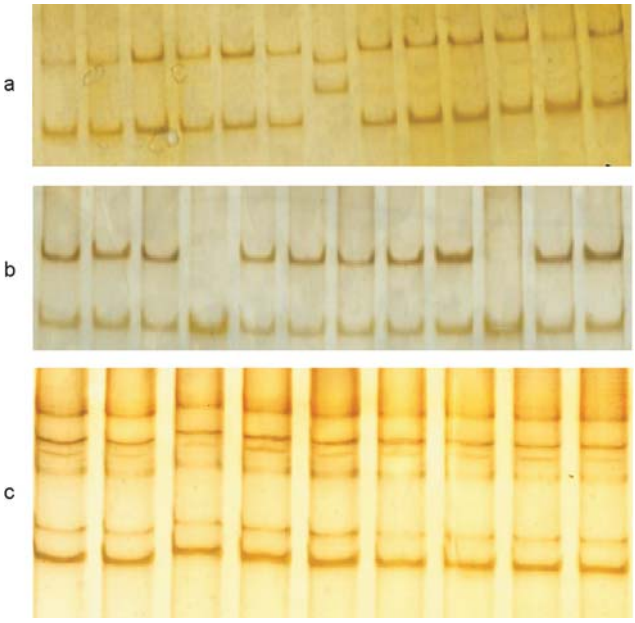


Fig. 1. SSCP patterns of three fragments of ovine AR gene. AR fragment I (a) and II (b): two types of banding patterns and AR fragment III (c): no polymorphism.

association with cryptorchism. However, the distribution of the banding pattern was significantly different for Mandya and Hassan sheep breeds thus contributing to the genomic variation between these breeds (Table.2). The sequencing of the fragment revealed a silent mutation of cytosine to thymine at 603 of Ovine AR exon1 sequence and no polymorphism at CAG-III repetitive site. The PCR-SSCP of the third fragment was monomorphic indicating no polymorphism in the GGC region of the AR gene (Fig.1.c).

AR is a member of the steroid hormone receptor family of genes. The first exon codes for the N-terminal domain (NTD) that is the transcriptional regulatory region of the protein. The NTD region displays high variability especially in trinucleotide repeats. The variation in CAG repeat length are reported to be associated with several disease conditions in humans (Almeida *et al.* 2013, Ryan and Crespi 2013), dog (Akitsugu *et al*. 2011) and pig (Trakooljul *et al.* 2004). But interestingly, no polymorphism at any of the trinucleotide repetitive region of sheep AR gene was observed in our study.

Trinucleotide repeats especially CAG length have evolutionary significance. The length of CAG repeats of

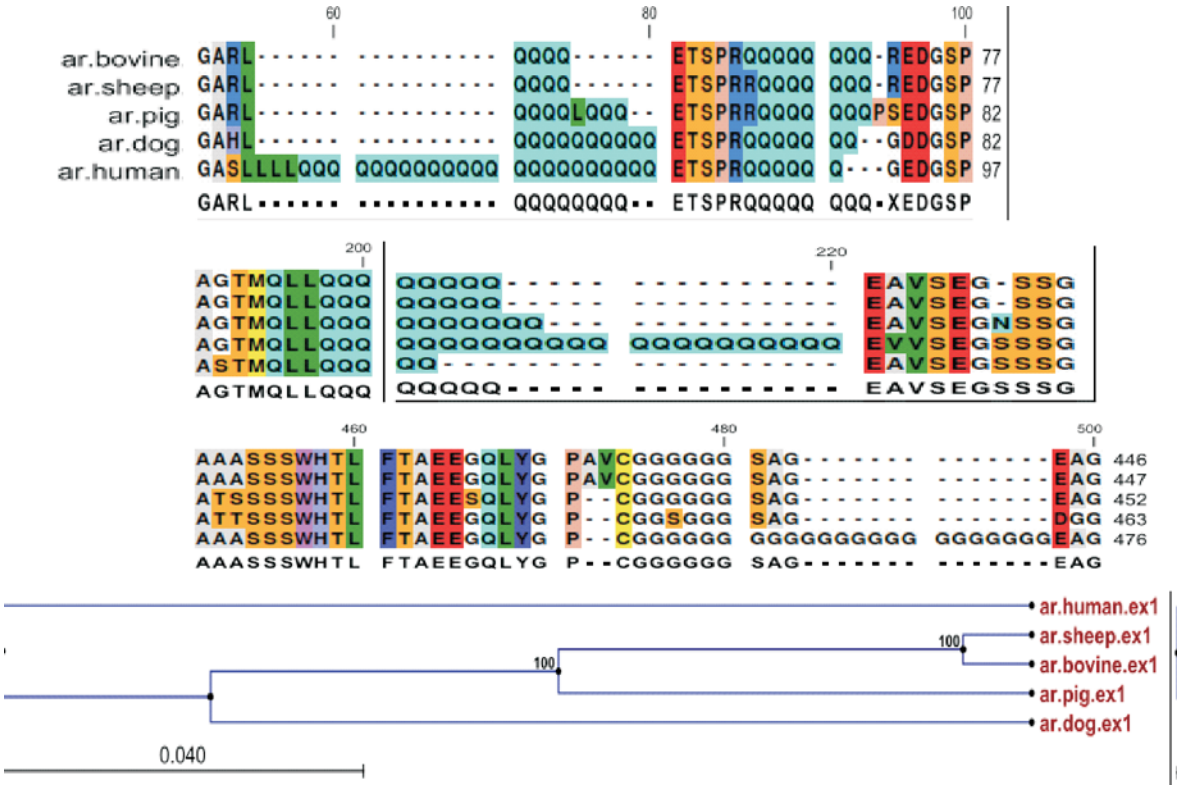


Fig. 2. Sequence alignment of trinucleotide regions of androgen receptor and phylogenetic tree.

(Fig.1.b). The sequence (Genbank KC878301.1) revealed that these banding patterns are not due to variation in CAG-III repeat number but due to a novel SNP, i.e. guanine to cytosine at 362 bp of exon1. The SNP is silent with no alterations in amino acid coded. The 362G was more frequent than 362C and the SNP did not show significant

AR gene in different primate species decreases with evolutionary distance from humans and postulated to have developmental or behavioral implications for the most advanced species (Hong *et al.* 2006). This was observed to be true for CAG-I length where in the length was highest in humans followed by dog, pig and sheep. Phylogenetic

analysis based on the repetitive regions of exon1 also revealed that bovines are closely related to sheep, followed by pig, dog and human (Fig. 2).

The strong selection mutation balance acts as the force in evolution and maintenance of variability at CAG repeats, thus, the variability in CAG repeats (Li *et al*. 2012), therefore the extent of variation in CAG repeats of a gene indirectly indicates the selection pressure on that gene. The present observation of no variability of CAG repeats in Ovine AR gene may indicate higher selection pressure on the gene i.e., males are intensely selected in sheep. Similarly Whan *et al.* (2010) have reported that the long history of cattle domestication involving intensive sire-based selection, primarily focused on growth and reproductive traits, may have selected against variation in the AR gene in cattle.

In conclusion, the CAG and GGC repetitive regions are non polymorphic and conserved in sheep population. Neither the trinucleotide repetitive regions nor the polymorphism in exon1 of ovine AR gene were involved in cryptorchidism.

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