RESEARCH ARTICLE

cDNA cloning, structural analysis, SNP detection and tissue expression profile of the *IGF1* gene in Malabari and Attappady Black goats of India

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Short Running title:-

Cloning, SNP detection and tissue expression profile of the caprine *IGF1* gene

Keywords : gene cloning; gene expression; goat; IGF1; mRNA; SNP

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Abstract

The Insulin-Like Growth Factor 1 (IGF1) plays an important role in growth, reproduction, foetal development and cell proliferation. The present study was conducted to clone and sequence the full-length coding sequence of the caprine *IGF1* gene from Attappady Black and Malabari breeds, two indigenous goat breeds of South India, to analyse its structure, and to ascertain the relative abundance of *IGF1* mRNA in different tissues. The caprine *IGF1* cDNA (GenBank Accession No: KJ549851 and KJ549852) contained a 465 bp ORF encoding IGF1 protein with 154 amino acid residues. A novel SNP was detected in the 3'UTR region, g.931A>G. Genotyping was performed in 277 goats from the two genetic groups using the PCR-Single Strand Conformational Polymorphism (SSCP) and two genotypes, AA and AG were observed at this locus. IGF1 is secretary pathway protein with 49 amino acid long signal peptide with 19 phosphorylation sites. Caprine IGF1 amino acid sequence was 83–99% identical to other species, with highest identity with the ruminants. Relative expression of *IGF1* was highest in uterus and

liver (P<0.05), followed by oviduct and muscle. This work provided an important experimental basis for further research on the functions of IGF1 in goats.
Keywords : gene cloning; gene expression; goat; IGF1; mRNA; SNP

Introduction

The Insulin-Like Growth Factor 1 (IGF1), an important component of somatotropic axis plays an important role in growth, reproduction, foetal development and cell proliferation. In the development and growth of muscles IGF1 acts as a mitogen and a differentiation factor by controlling the mitogenic and myogenic processes during muscle development (Ewton *et al.* 1994; Florini *et al.* 1996; Davis and Simmen 2006). The role of IGFs in reproduction was reported by Pushpakumara *et al.* (2002) as IGFs influence the early embryonic development by acting directly on the embryo or indirectly by modulation of oviductal secretions and muscular activity. IGF1 stimulates ovarian function, steroidogenesis and folliculogenesis (Lucy 2000; Behl and Kaul 2002; Spicer *et al.* 2002), also associated with gestation length (Sirotkin *et al.* 2003), other reproductive traits (Echternkamp *et al.* 2004; Wang *et al.* 2011), embryo development (Velazquez *et al*. 2005) and conception rate (Patton *et al*. 2007). Apart from the role in growth and reproduction, IGF1 stimulates glucose absorption, lipogenesis, myogenesis and progesterone synthesis in granulose cells, inhibition of cell death, activation of cell cycle genes etc. (Reyna *et al.* 2010). A few molecular researches were available for goats regarding the association of *IGF1* with growth (Liu *et al.* 1993; Arends *et al.* 2002; Machado *et al.* 2003; Zhang *et al.* 2008; Sharma *et al.* 2013) and reproductive traits (Ge *et al.* 2001; Estany *et al*. 2007; Wang *et al.* 2011; He *et al.* 2012; Thomas *et al.* 2016). As IGF1 plays crucial role in almost all body functions, including growth, reproduction and metabolism, the current research was designed to investigate the nucleotide sequences, base mutations and to ascertain the mRNA expression levels of *IGF1* gene in different tissues, (viz., muscle, uterus, oviduct and liver) in Malabari goats, a native Indian breed well known for its high prolificacy and growth rate and in Attappady Black goats which is highly adapted to hot climate and hilly ecological conditions in the Attappady region of Palakkad district of Kerala, South India.

Materials and methods

Sample collection, RNA and DNA isolation and first strand cDNA synthesis

All experimental procedures were performed according to the guidelines of the Institutional Animal Ethics Committee of Kerala Veterinary and Animal Sciences University. Tissue samples of oviduct, uterus, liver and muscle (approximately 100 mg each) were collected from six adult animals (in the middle of the estrus) each from the Malabari and Attappady Black female goats from Kerala Veterinary and Animal Science University Meat Plant, immediately immersed in RNAlater® (Sigma-Aldrich), total RNA was extracted from the tissue samples using the Gen Elute mammalian total RNA miniprep kit (RTN10, Sigma Aldrich), RNA samples were quantified by NanoDrop spectrophotometer (Thermo Scientific, USA) and checked for the integrities on 0.8% agarose gel. Reverse transcription was performed to synthesise cDNA from isolated RNA using the RevertAid first strand cDNA synthesis kit (Thermo Scientific, K1622). A total number of 277 goats (2-5 years of age), which are genetically unrelated belonging to the Malabari (n = 175) and Attappady Black breeds (n = 102) were used for polymorphism analysis of *IGF1* gene. Venous blood (6 mL) was collected from the jugular vein of each animal and stored at 4 °C until processing. The genomic DNA from the white blood cells was extracted employing the standard phenol chloroform method.

PCR, Molecular Cloning and Sequence analysis

cDNA was amplified using primers designed from the available *IGF1* mRNA sequence in the GenBank (Accession No. D11378) using Primer3 software (table 1). The PCR for the amplification of the *IGF1* gene was performed in 50 µL reaction volume containing 100 ng cDNA, 5 µL of 10X Buffer, 1µL of 0.2 mM dNTP, 10 pM each of forward and reverse primers and 1 µL of JumpStart AccuTaq LA DNA Polymerase (2.5U/µL) with proof reading activity (Sigma Aldrich). The cycling protocol was 96ºC for two min, 35 cycles of 95ºC for 30 s, 57ºC for 20 s, 68ºC for one min and a final extension at 68ºC for 5 min. The products were analyzed on 1% agarose gel electrophoresis. Gel purified PCR products were cloned into pGEM-T Easy Vector (Promega, USA), transformed into DH5α strain of *Escherichia coli* and the clones harboring the *IGF1* was confirmed by blue white screening, colony PCR, plasmid PCR and restriction enzyme digestion (EcoRI) of plasmids. The plasmid was extracted from the clones

using PureLink® Quick Plasmid Miniprep Kit (Invitrogen) and sequenced (Xcelris, Ahmedabad), using T7 and SP6 primers. Sequence data obtained were edited manually using Chromas Lite Ver.2.33, (http//www.technelysium.com.au/chromas.html) and subjected to BLASTn analysis (www.ncbi.hlm.nih.gov/BLASTn) to retrieve similar sequences of 16 other species including artiodactyl, mammalian and avian origin. The various bioinformatics tools were used for the sequence analysis include, ORF finder, EXPASY translate tool, Clustal omega, SWISSMODEL server, self-optimized prediction method (SOPMA), ProtParam tool, TargetP 1.1, SignalP 4.1, NetPhos 2.0, NetPhosK 1.0, NCBI conserved domain search and DNAstar Lasergene MegAlign program.

PCR-SSCP and SNP identification

Cloning and sequencing of *IGF1* cDNA from both breeds revealed a novel SNP (g.931A>G) in the 3' UTR region of the Malabari breed. Primers were designed as previously described, to perform PCR (Table 1) for a 269 bp fragment of *IGF1* gene, to characterize the detected SNP. The 25 µL reaction volume contained 50 ng genomic DNA, 2.5 µL 10X reaction buffer, 0.2 mM dNTP, 10 pM of each primer and 0.5 U of Taq DNA polymerase. The cycling protocol was 3 min at 95°C, 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 20 s, extension at 72 °C for 30 s with a final extension at 72°C for 5 min and PCR was conducted in a thermal cycler (Biorad, USA). SSCP was performed to genotype the 277 genetically unrelated goats belonging to Malabari and Attappady Black. The 5 µL PCR products were mixed with a 10 µL denaturing dye (9.5 mL formamide deionised, 0.4 mL of 0.5M EDTA, 2.5 mg xylene-cyanole and 2.5 mg bromophenol blue), centrifuged, denatured by keeping at 95 ºC for 10 minutes and snap chilled immediately on ice for 10 minutes. Denatured PCR products were separated by polyacrylamide gel electrophoresis (acrylamide:bisacrylamide = 29:1). The 12% polyacrylamide gel was processed at 140V for 17 hours at 4ºC, in a vertical electrophoresis apparatus (Hoefer, USA). The SSCP patterns were visualised using silver nitrate staining, photographed and analysed. Representative PCR products from different genotypes were sequenced to detect variations in nucleotides and aligned with other sequences in GenBank employing BLASTn and EMBOSS Needle.

Quantitative real-time PCR (RT-qPCR)

The relative quantification of *IGF1* mRNA expression was done by Real Time quantitative PCR (RTq-PCR) in different goat tissues, *viz*, muscle, liver, uterus and oviduct. Six samples from each tissue with three technical replicates per sample were analysed and the relative expression of mRNA were normalised using *GAPDH* gene. Primer pairs for caprine *IGF1* and *GAPDH* were designed using Primer3 software (table 1). RTq-PCR was conducted in a 25 µL reaction volume containing 50 ng of cDNA and 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The reactions were performed using cycling values of 95 ºC for three min followed by 40 cycles of 95 ºC for 30 s, 60 ºC for 15 s, 72 ºC for 30s and 95 ºC for 15 s, 60 ºC for 15 s and 95 ºC for 15 s. In addition, the excepted size of the amplicon and the absence of nonspecific products were confirmed by 2% agarose gel electrophoresis. Melt curve analysis was performed and the relative expression of *IGF1* mRNA was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). To control for false positive reactions, the reverse transcriptase-negative control for each template and non template control for each primer pair were run. Statistical comparison between samples was performed using ANOVA (Analysis of variance) and independent sample t-test (SPSS V.21).

Results and discussion

The 974 bp sequence of the *IGF1* gene in Malabari and Attappady Black goats (GenBank accession No. KJ549851 and KJ549852), contained a predicted ORF of 465 bp encoding a protein of 154 amino acid residues with a calculated molecular weight of 17.08 KDa and isoelectric point of 9.36. One novel SNP was detected in the 3'UTR region, g.931A>G in Malabari breed. The SSCP analysis of 269 bp fragment of 3' UTR of *IGF1* displayed a polymorphic band pattern, sequencing of the PCR products revealed two genotypes with one mutation (A→G transition at 931th position). The sequencing maps for the SNPs for AA and AG genotypes are depicted in figure 1. The polymorphisms at the 3' UTR may be within or near the miRNA binding site; so these polymorphisms may affect the miRNA function and lead to differential expression of genes, thereby affecting the phenotype (Trott *et al*. 2014). In the present study the frequency of AA genotype was 0.98 and AG genotype was 0.02 in the 277

genetically unrelated goats selected for the present study. Since the frequency of AG genotype was very low in the population, the association analysis with production traits could not be carried out. The effect on gene expression and association study of the detected SNP should be carried out in a larger goat population. The amino acid sequence analysis of IGF1 revealed that IGF1 is secretary pathway protein with 49 amino acid signal peptide with 19 phosphorylation sites, 5 serine, 3 threonine, 2 tyrosine and 9 specific protein kinase phosphorylation loci (figure 2). The conserved domain was predicted between 50^{th} and 114^{th} position of peptide sequence. The number of negatively charged residues (Asp + Glu) was 11 and the number of positively charged residues (Arg+Lys) was 22; the instability index (II) was computed to be 54.51. Hydrophobic correlation analysis showed that the grand average of hydropathicity (GRAVY) was -0.267. The predicted amino acid sequences of *IGF1* gene was subjected to MSA using Clustal omega and MegAlign (Lasegene software, DNASTAR) program to know the homology and divergence with that of 16 other species. IGF1 protein sequence revealed 154 amino acids peptide for ruminants and one less ie.,153 amino acids for the other species, which indicated that the amino acid sequences between goat and other ruminants may have similar biological functions *ie,* may be due to highly conserved nature of IGF1 sequence (Wallis 2009; Philippou *et al.* 2014). The caprine sequence showed 83–99% similarity with other species. The caprine IGF1 protein shared highest homology with ruminants compared to other mammals and aves, which is evident in phylogenetic analysis (figure 3). Protein secondary structure showed that goat IGF1 protein has 51 alpha helices, 10 beta turns, 21 extended strands and 72 random coils. The predicted 3D structural model of the goat IGF1 protein (between amino acids 51 and 112) was similar to that of the structure of human IGF1 in the Protein Data Bank (PDB: 1wqj) (figure 4). The predicted 3D structure will provide the basis for further structure–function studies of IGF1.

The *IGF1* mRNA was highly expressed in uterus and liver ($P<0.05$), oviduct and muscle (figure 5). All the tissues studied showed a moderate to high level of *IGF1* mRNA expression, indicating role of this growth factor in almost all body functions. IGF1 is synthesized as an endocrine hormone and act on target tissues in a paracrine/autocrine fashion (Florini *et al.* 1996; Laron 2001). The expression of *IGF1* mRNA was highest in the uterus indicates its decisive role in embryonic implantation and development (Singer *et al.* 2014). Between breed comparison

indicated a significantly higher of *IGF1* expression in muscles of Malabari than Attappady Black breed. During postnatal skeletal muscle growth *in vivo* or in fully differentiated muscle cells, IGFs stimulate the rate of protein synthesis and inhibits the rate of protein degradation thereby enhancing myofibre hypertrophy and increase in myofibre diameter which may affect muscle tenderness (Florini *et al.* 1996; Koohmaraie *et al.* 1995), so association of *IGF1* expression and meat quality studies are necessary to confirm the effect of higher expression of *IGF1* mRNA in muscles of Malabari breed. Further earlier researches suggest that IGF1 deficiencies causes pronounced growth retardation and delayed onset of puberty (Baker *et al.* 1993; Laron 2001; Estany *et al.* 2007).

In conclusion, the SNP observed in this study was in the 3'UTR region of *IGF1* gene may be within or near the miRNA binding site; which may interfere with miRNA function and lead to differential expression of genes, which provides a theoretical basis for future research into the function of this SNP. Highest mRNA expression in the uterine tissues is indicative of the role of *IGF1* in prenatal development of foetus. Breed difference in the skeletal muscle tissue expression of *IGF1* mRNA is suggestive of its role in meat quality. So the physiological and biochemical functions, together with the observations obtained in our study, indicate that the *IGF1* gene might play important roles in almost all body functions especially growth, reproduction and meat quality traits in goats. Further studies are required to establish the role of the detected SNP in the *IGF1* gene expression and association with economic traits in goats. The results of the present study, suggests that *IGF1* could be considered as a potent candidate gene for Marker Assisted Selection in goats.

Conflicts of interest

The authors report no conflicts of interest to disclose.

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Table 1. Primer sequences and information used in the present study

Primer name	Primer sequence (5'-3')	Product size	T_A (°C)	Accession Number
IGF1UTRF	TCTTTAGGAGTGATTGTTCAAAGC	269bp	58	KJ549851
IGF1UTRR	TTTGCGTAGAAAGAAGTGCAAA			
IGF1CDF	TCCCATCTCCCTGGATTTC	974bp	57	D11378
IGF1CDR	TTTTTGCGTAGAAAGAAGTGC			
IGF1RTF	CATCCTCCTCGCATCTCTTC	111bp	60	D11378

IGF1RTR	ACTGGAGAGCATCCACCAAC			
GAPDHF	TGGAGAAACCTGCCAAGTATG	127bp	60	XM_005680968
GAPDHR	TGAGTGTCGCTGTTGAAGTC			

Figure 1. PCR-SSCP pattern and sequence maps of AG and AA genotypes with g.931A>G locus at the 3' UTR of goat *IGF1* gene

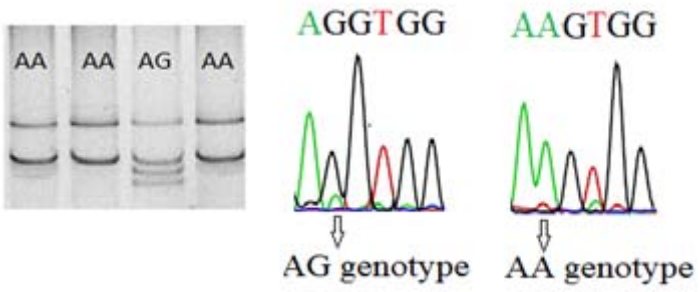


Figure 2. ORF and deduced amino acid sequences of Malabari and Attappady Black goat IGF1. * indicates the termination codon; the underlined area indicates the signal peptide. The bold characters indicate predicted phosphorylation sites (Ser, Thr, Tyr).

```
atgggaaaaatcagcagtcttccaacccaattatttaagtgctgcttttgtgatttcttg
 M  G  K  I  S  S  L  P  T  Q  L  F  K  C  C  F  C  D  F  L
aagcaggtgaagatgccagtcacatcctcctcgcatctcttctatctggccctgtgcttg
 K  Q  V  K  M  P  V  T  S  S  S  H  L  F  Y  L  A  L  C  L
ctcgccttcaccagctctgccacggcgggacccgagaccctctgcggggctgagttggtg
 L  A  F  T  S  S  A  T  A  G  P  E  T  L  C  G  A  E  L  V
gatgctctccagttcgtgtgtggagacaggggctttatttcaacaagcccacggggtac
 D  A  L  Q  F  V  C  G  D  R  G  F  Y  F  N  K  P  T  G  Y
ggctcgagcagtcggagagcgccccagacaggaatcgtggatgagtgctgcttccggagc
 G  S  S  S  R  R  A  P  Q  T  G  I  V  D  E  C  C  F  R  S
tgtgatctgaggaggctggagatgtactgtgcgcctctcaagcccaccaagtcagcccgc
 C  D  L  R  R  L  E  M  Y  C  A  P  L  K  P  T  K  S  A  R
tcagtccgtgcccagcgccacaccgacatgcccaaggctcagaaggaagtacatttgaag
 S  V  R  A  Q  R  H  T  D  M  P  K  A  Q  K  E  V  H  L  K
aacacaagtagagggagtgcaggaaacaagaactacagaatgtag
 N  T  S  R  G  S  A  G  N  K  N  Y  R  M  *
```


Figure 3. Phylogenetic tree constructed from sequence data of Malabari goat *IGF1* cDNA and 16 different species, showing highly conserved nature of *IGF1* among ruminants

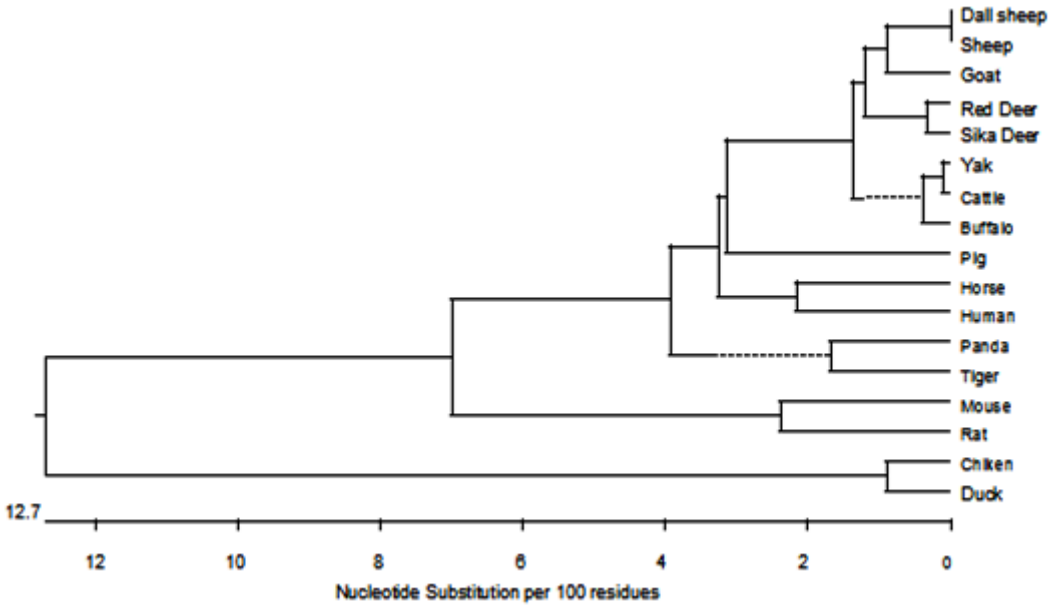
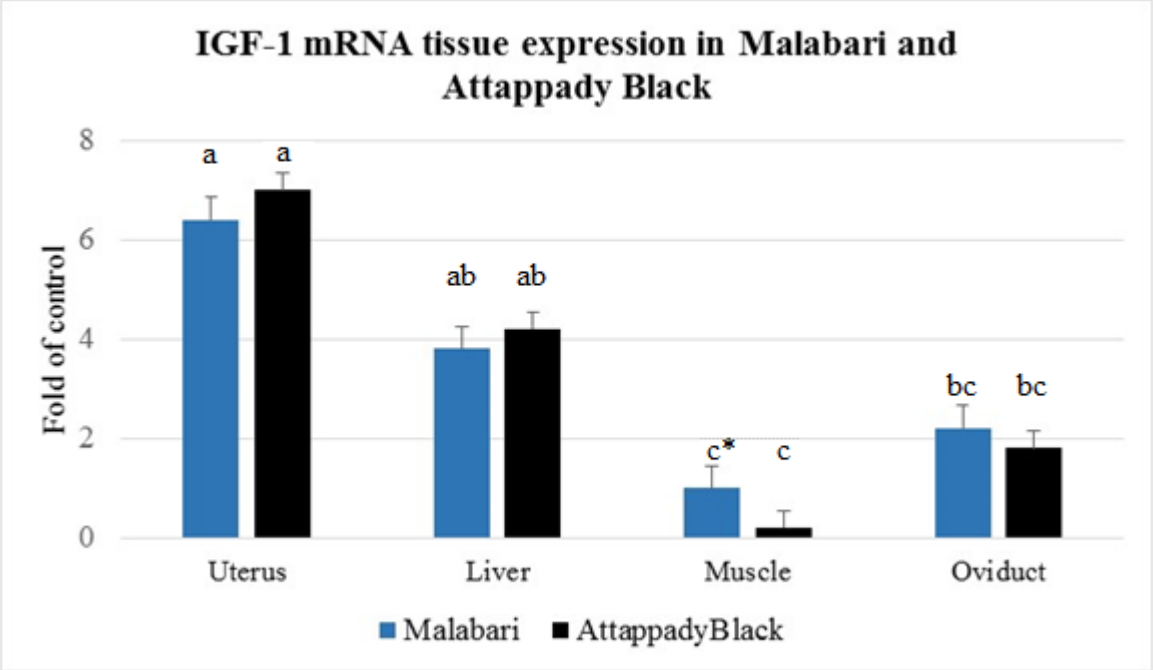


Figure 4. Three-dimensional structural model of caprine IGF1 (residues 51–112) based on homology modeling.

Model information:
Modelled residue range: 51 to 112
Based on structure: [1wqj]
Sequence Identity [%]: 100%

Figure 5. Differential tissue expression of *IGF1* mRNA in Malabari and Attappady Black goats indicating moderate to high level of expression (For different superscripts $P<0.05$).The fold changes in the relative expression were normalised by the relative expression of the *GAPDH* mRNA (reference gene). * indicates the difference in relative expression levels of *IGF1* mRNA in the muscle tissues of two genetic groups ($P<0.05$).


IGF-1 mRNA tissue expression in Malabari and Attappady Black
Fold of control
8
6
4
2
0
a
a
ab
ab
c*
c
bc
bc
Uterus
Liver
Muscle
Oviduct
Malabari
AttappadyBlack