RESEARCH ARTICLE

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

THOMAS NAICY[1]*, THIRUPATHY VENKATACHALAPATHY[2], THAZHATHUVEETTIL ARAVINDAKSHAN[2], KUNNIYOOR CHEEMANI RAGHAVAN[2], MANGATTUMURUPPEL MINI[3] and KULANGARA SHYAMA[4]

[1]*Department of Animal Genetics and Breeding, CVAS, Pookode, Wayanad 673 576, India*
[2]*Centre for Advanced Studies in Animal Genetics and Breeding, Mannuthy 680 651, India*
[3]*Department of Veterinary Microbiology, and* [4]*Department of Animal Nutrition, CVAS, Mannuthy 680 651, India*

## Abstract

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 nos: KJ549851 and KJ549852) contained a 465-bp open reading frame 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 a 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.

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## Introduction

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.* 2008) 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 on 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, (namely 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.

*For correspondence. E-mail: naicy@kvasu.ac.in.

**Keywords.** gene cloning; gene expression; goat; insulin-like growth factor 1; mRNA; single-nucleotide polymorphism.

## Materials and methods

### *Sample collection, RNA and DNA isolations, 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 (~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, Bengaluru, India), total RNA was extracted from the tissue samples using the Gen Elute mammalian total RNA miniprep kit (RTN10, Sigma-Aldrich, Bengaluru, India), RNA samples were quantified by NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA) and checked for the integrities on 0.8% agarose gel. Reverse transcription was performed to synthesize cDNA from isolated RNA using the RevertAid first strand cDNA synthesis kit (K1622, Thermo Scientific, Waltham, USA). 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 $\mu$L reaction volume containing 100 ng cDNA, 5 $\mu$L of 10× Buffer, 1 $\mu$L of 0.2 mM dNTP, 10 pM each of forward and reverse primers and 1 $\mu$L of JumpStart AccuTaq LA DNA polymerase (2.5 U/$\mu$L) with proofreading activity (Sigma-Aldrich, Bengaluru, India). The cycling protocol was 96°C for 2 min, 35 cycles of 95°C for 30 s, 57°C for 20 s, 68°C for 1 min and a final extension at 68°C for 5 min. The products were analysed on 1% agarose gel electrophoresis. Gel-purified PCR products were cloned into pGEM-T Easy Vector (Promega, Wisconsin, USA), transformed into DH5$\alpha$ strain of *Escherichia coli* and the clones harbouring the *IGF1* was confirmed by blue white screening, colony PCR, plasmid PCR and restriction enzyme digestion (*Eco*RI) of plasmids. The plasmid was extracted from the clones using PureLink® Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, USA) and sequenced (Xcelris, Ahmedabad, India), 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 origins. Various bioinformatics tools were used for the sequence analysis which includes 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.

### *Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and single-nucleotide polymorphism (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 $\mu$L reaction volume contained 50 ng genomic DNA, 2.5 $\mu$L 10× 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 $\mu$L PCR products were mixed with a 10 $\mu$L denaturing dye (9.5 mL formamide deionized, 0.4 mL of 0.5 M EDTA, 2.5 mg xylene–cyanole and 2.5 mg bromophenol blue), centrifuged, denatured by keeping at 95°C for 10 min and snap-chilled immediately on ice for 10 min. Denatured PCR products were separated by polyacrylamide gel

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

| Primer name | Primer sequence (5′–3′) | Product size (pb) | $T_a$ (°C) | Accession number |
|---|---|---|---|---|
| IGF1UTRF | TCTTTAGGAGTGATTGTTCAAAGC | 269 | 58 | KJ549851 |
| IGF1UTRR | TTTGCGTAGAAAGAAGTGCAAA | | | |
| IGF1CDF | TCCCATCTCCCTGGATTTC | 974 | 57 | D11378 |
| IGF1CDR | TTTTTGCGTAGAAAGAAGTGC | | | |
| IGF1RTF | CATCCTCCTCGCATCTCTTC | 111 | 60 | D11378 |
| IGF1RTR | ACTGGAGAGCATCCACCAAC | | | |
| GAPDHF | TGGAGAAACCTGCCAAGTATG | 127 | 60 | XM_005680968 |
| GAPDHR | TGAGTGTCGCTGTTGAAGTC | | | |

electrophoresis (acrylamide : bisacrylamide = 29 : 1). The 12% polyacrylamide gel was processed at 140 V for 17 h at 4°C in a vertical electrophoresis apparatus (Hoefer, USA). The SSCP patterns were visualized 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.

***RT-qPCR***

The relative quantification of *IGF1* mRNA expression was done by real-time quantitative PCR (RTq-PCR) in different goat tissues, namely 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 normalized using *GAPDH* gene. Primer pairs for caprine *IGF1* and *GAPDH* were designed using Primer3 software (table 1). RTq-PCR was conducted in a 25 $\mu$L reaction volume containing 50 ng of cDNA and 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The reactions were performed using cycling values of 95°C for 3 min followed by 40 cycles of 95°C for 30 s, 60°C for 15 s, 72°C for 30 s and 95°C for 15 s, 60°C for 15 s and 95°C 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 the false positive reactions, the reverse transcriptase-negative control for each template and nontemplate control for each primer pair were run. Statistical comparison between samples was performed using analysis of variance (ANOVA) and independent sample *t*-test (SPSS ver. 21).

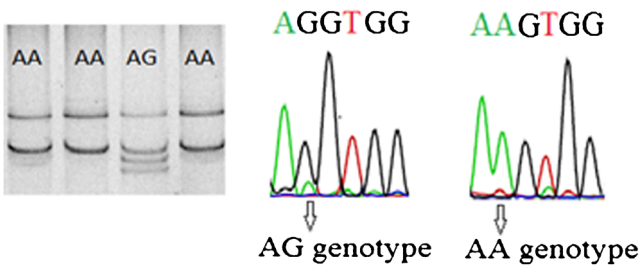


**Figure 1.** PCR-SSCP pattern and sequence maps of AG and AA genotypes with g.931A>G locus at the 3′UTR of goat *IGF1* gene.

## Results and discussion

The 974-bp sequence of the *IGF1* gene in Malabari and Attappady Black goats (GenBank accession nos. KJ549851 and KJ549852) contained a predicted open reading frame (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; thus 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, five serine, three threonine, two tyrosine and nine specific protein kinase

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

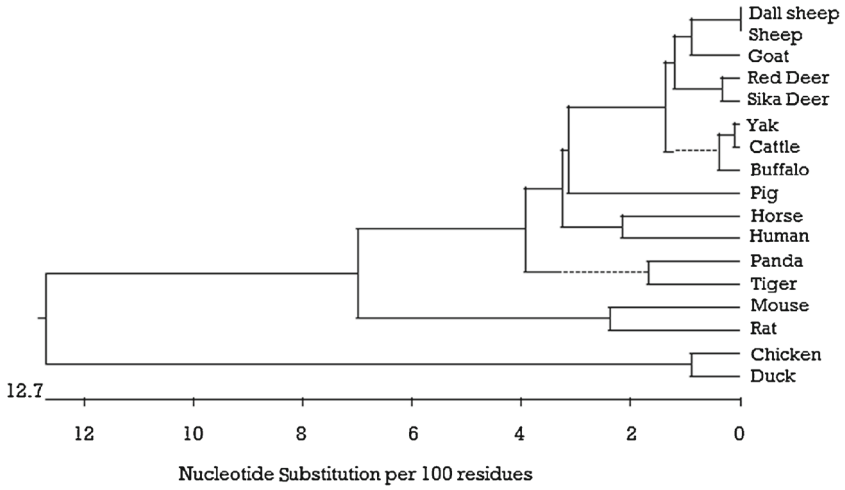


**Figure 3.** Phylogenetic tree constructed from sequence data of Malabari goat *IGF1* cDNA and 16 different species, showing highly conserved nature of *IGF1* among ruminants.

phosphorylation loci (figure 2). The conserved domain was predicted between 50th and 114th positions 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 multiple sequence alignment (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 i.e., 153 amino acids for the other species, which indicated that the amino acid sequences between goat and other ruminants may have similar biological functions i.e., 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.

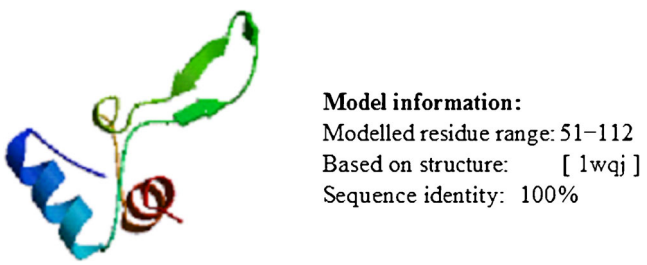


**Figure 4.** Three-dimensional structural model of caprine IGF1 (residues 51–112) based on homology modelling.

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 indicates a significantly higher *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

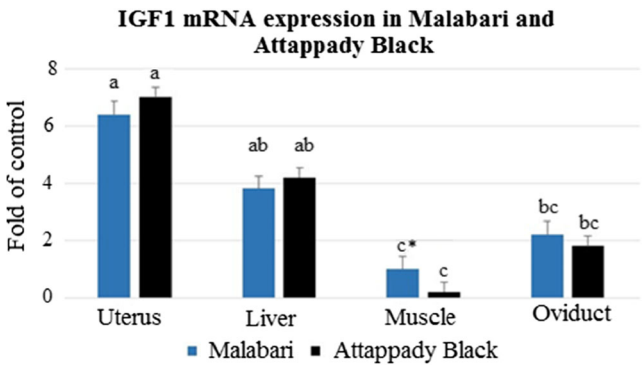


**Figure 5.** Differential tissue expression of *IGF1* mRNA in Malabari and Attappady Black goats indicating moderate to high levels of expression (for different superscripts $P < 0.05$). The fold changes in the relative expressions were normalized by the relative expression of the *GAPDH* mRNA (reference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

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 (Koohmaraie *et al.* 1995; Florini *et al.* 1996), thus 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. Thus, 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.

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