Theriogenology 86 (2016) 2172–2178

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Nerve Growth Factor gene ovarian expression, polymorphism identification, and association with litter size in goats

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form 12 July 2016
Accepted 12 July 2016

Keywords:
Attappady Black
Malabari
Gene expression
Nerve Growth Factor
Litter size
single-nucleotide polymorphism

ABSTRACT

The *Nerve Growth Factor* (NGF) plays an important role in reproduction by augmenting folliculogenesis. In this study, the coding regions of caprine *NGF* gene were analyzed to detect single-nucleotide polymorphisms (SNPs), their association with litter size, and the relative ovarian expression of *NGF* gene in the two indigenous goat breeds of South India *viz*., the prolific Malabari and less-prolific Attappady Black. The sequence analysis of the third exon containing the entire open reading frame of *NGF* gene was observed to be of 808 bp with one nonsynonymous mutation at 217th position. Later, polymerase chain reaction (PCR) was performed to amplify a region of 188 bp covering the region carrying the detected mutation. The genomic DNAs from the goats under study (n = 277) were subjected to PCR and single strand conformation polymorphism (SSCP). On analysis, four diplotypes *viz*., AA, AB, AC, and AD were observed with respective frequencies of 0.50, 0.22, 0.27, and 0.01. Sequencing of the representative samples revealed an additional synonymous mutation, i.e., g.291C>A. Statistical analysis indicated that *NGF* diplotypes and the SNP g.217G>A were associated with litter size in goats ($P < 0.05$). Relative expression of *NGF* gene was significantly higher in the ovaries of goats with history of multiple than single births ($P < 0.05$). The results of the present study suggest the significant effect of the *NGF* gene on litter size in goats and identified SNPs would benefit the selection of prolific animals in future marker-assisted breeding programs. The two novel PCR-restriction fragment length polymorphisms designed, based on the detected SNPs, would help in the rapid screening of large number of animals in a breeding population for identifying individual animals with desired genetic characteristics.

1. Introduction

Research on the identification of genes and their association with economic traits in farm animals could assist in the genetic selection of breeding stock. Litter size at birth is one of the most important reproductive traits for the selection of multiparous animals [1,2]. Superior kidding rate is a very important economic trait in goat production and ovulation rate is the precondition of kidding rate [3], and improving these traits in indigenous goat breeds may increase the profits from goat rearing. Because the heritability of the reproductive traits is low, traditional selection methods can be complemented with marker-assisted selection, to increase the intensity of

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http://dx.doi.org/10.1016/j.theriogenology.2016.07.011

selection [4]. The influence of candidate genes on reproductive traits differs considerably because these genes could affect the physiological pathways, metabolism, and phenotypic expression differentially with different paracrine or autocrine effects. The major focus of goat breeding for genetic improvement greatly relies on candidate genes influencing reproductive traits so as to ascertain the functions of genes causing changes in phenotypic values of the trait, molecular structure analysis, expression profile, sequence variability, and their association with phenotypes. The selection for favorable alleles of candidate genes could help in the development of a breeding stock of goats with high litter size [5–7].

Nerve Growth Factor (*NGF*) is a member in the neurotrophin family [8], whose major site of synthesis and secretion is ovarian cells [9,10], and can be considered as a key regulator of ovarian function because it promotes the development of preantral follicles by the cell-specific activation of TrkA receptors in the preovulatory follicles and influences the process of ovulation [11]. Its role in reproduction was demonstrated experimentally in *NGF* null mutant mice, which exhibited a markedly reduced numbers of primary and secondary follicles even at the normal serum gonadotrophin levels [12]. The *NGF* promotes the production of a principal angiogenic factor, vascular endothelial growth factor (VEGF) from the developing follicles, granulosa cells, and CL leading to enhanced ovarian follicular development and luteogenesis. It was also reported that any change in *NGF* expression could result in ovarian disorders related to impaired angiogenesis [13]. An upregulation of *NGF* in the ovary promotes steroidogenesis by enhancing the expression of enzyme genes involved in 17α-hydroxyprogesterone, testosterone, and estradiol synthesis [14]. In spite of the clear elucidation of the role of *NGF* gene in influencing the reproductive performance, the evaluation of this as a candidate gene for genetic selection of breeding animals has not been conducted extensively and systematically. Recently, it was reported that *NGF* gene demonstrated highest expression in caprine reproductive tissues, and a synonymous mutation in the coding region of this gene was associated with litter size in goats [15]. The present study envisages the characterization of coding region of caprine *NGF* gene, from two distinct south Indian indigenous goat breeds *viz*., the prolific Malabari and less-prolific Attappady Black, to detect the genetic variants and their association with prolificacy, if any. In addition, the expression of *NGF* gene in the ovaries of these two breeds was also quantified to know the *in vivo* influence of this gene in prolificacy.

2. Materials and methods

To characterize and identify the genetic variants of caprine *NGF* gene from Malabari and Attappady Black, genomic DNA was isolated from 277 goats, further subjected to polymerase chain reaction (PCR), molecular cloning, and SSCP. The total RNA was isolated from ovarian tissues from goats with multiple and single birth history for the three previous kiddings from Malabari and Attappady Black goats, respectively, for the *NGF* mRNA expression analysis by quantitative real-time (qRT) PCR.

2.1. Animals and sample collection for DNA and RNA isolation

A total of 277 female goats (2–5 years of age) belonging to the Malabari (n = 175) and Attappady Black breeds (n = 102) were included in the present research from four centers of three districts of south Indian state, Kerala, *viz*., Thrissur, Malappuram, and Palakkad. The goats were selected at random after verifying the farm records so as to make the individuals to be as unrelated as possible. The mean litter size of the selected animals belonging to each breed is provided in Supplementary Table 1. 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 using the standard phenol chloroform method.

The ovarian tissue samples were collected from six goats each with multiple and single birth history for the three previous kiddings from Malabari and Attappady Black goats, respectively, from the Kerala Veterinary and Animal Science University Meat Plant. Approximately, 100 mg of ovarian tissues were collected, without including the follicles or their parts, and immediately immersed in RNAlater (Sigma–Aldrich) and stored at −80 °C until the isolation of RNA. Total RNA from the tissue samples was extracted using the Gen Elute mammalian total RNA miniprep kit (RTN10, Sigma–Aldrich) and treated with DNaseI (DNase1 kit, Sigma–Aldrich) to prevent genomic DNA contamination. The quantification of RNA was done by NanoDrop spectrophotometer (Thermo Scientific, USA). The mean ratios of RNA at A260/A280 and A260/A230 were 2.02 ± 0.17 and 2.13 ± 0.15, respectively. Furthermore, the integrity of extracted RNA was verified using 0.8% agarose gel electrophoresis to observe clear bands of 28S and 18S rRNA, indicating its high quality. Subsequently, cDNAs were synthesized using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific, K1622) with 1 μg of RNA in a reaction volume of 40 μL and were stored at −80 °C until use.

2.2. NGF gene exon 3 PCR amplification, molecular cloning, and sequence analysis

The caprine *NGF* gene exon 3 was amplified from genomic DNA using the published primers [15]. A 50 μL PCR reaction was performed with 100 ng of the extracted genomic DNA, 5 μL of 10X Buffer, 1 μL of 10 mM dNTP, 10 pM each of forward and reverse primers, and 1 μL of JumpStart AccuTaq LA DNA Polymerase (2.5U/μL) with proofreading activity (Sigma–Aldrich). The cycling protocol was 96 °C for 2 minutes, 35 cycles of 95 °C for 30 seconds, 58 °C for 20 seconds, 68 °C for 1 minute, and a final extension at 68 °C for 7 minutes. The amplicons were run on 1% agarose gel, purified by gel extraction kit (Life Technologies, USA), cloned into the pGEM-T Easy Vector (Promega, USA), and transformed into the *Escherichia coli* (DH5α strain). The clones harboring the *NGF* gene were selected by blue white screening and confirmed with colony PCR. The plasmids were extracted from the positive clones using the PureLink Quick Plasmid Miniprep kit (Invitrogen) and sequenced