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EFFECT OF G(129)R POLYMORPHISM IN GROWTH DIFFERENTIATION FACTOR 9 GENE ON AWASSI EWES THAT BREED OUT OF SEASON

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Abstract

Growth differentiation factor 9 (GDF9) is a member of the TGF β superfamily that plays a critical role in ovarian follicular development and ovulation rate. The recent study was performed to identify the linkage between GDF9 mutation (G(129)R) and seasonality in awassi sheep. Thirty mature non-pregnant ewes with were used in this study between September/ 2018 to January/2019. Fifteen ewes were lambed at September and Novamber/2018, which considered Seasonal group, and the Non-Seasonal group which 15 ewes lambed in mid-December and January/2019. Two primers were utilized to amplify exon I of GDF9 gene by polymerase chain reaction (PCR) after DNA extraction from blood specimens. Polymorphisms were revealed via sequencing and compared with the sequencing of the ovine GDF9 gene in NCBI data bases. The results revealed one single nucleotide polymorphism (SNP) G(129)R in chromosome 5 of *GDF9* gene (exon I) when compared with Sequence ID: FJ429111.1, without resulted in an amino acid. Two genotypes (GG and GA) were observed with higher significant differences ($P<0.01$) between genotypic frequencies of G(129)R locus. The results showed that mutant heterozygote genotype (GA) recorded highly significant increased ($P<0.001$) in non-seasonal ewes (60.00) as compared with wild homozygote (GG) genotype (40.00). In contrast, genotype frequency (GG) was recorded higher significantly increased ($P<0.001$) in seasonal ewes (63.00) when compared with (GA) genotype (37.00). As a conclusion, exon I polymorphisms of *GDF9* gene in Awassi breed have an expected effect on the Seasonality and the mutant genotype find majorly in out of Season awsassi ewes.

INTRODUCTION

The GDF9 and Bone morphogenetic proteins (BMPs) are part of transforming growth factors (β family) [1]. GDF9 and BMP 15 modulate other hormones and factors to control follicular growth and apoptotic signaling [2,3]. In addition, GDF9 activate follicular development, granulosa cells proliferation, and follicular luteinization and keep up typical follicular condition in ovine ovary [4]. The expression of GDF9 happens early in oocytes throughout folliculogenesis (from initial stage of follicular progression until ovulation) [5], therefore, GDF-9 acts as an intraovarian regulator for early antral follicle transition from pre-antral follicle [6].

Besides the GDF9 and BMP15 functions in cumulus cell expansion, oocyte maturation and ovulation [7, 8], GDF-9 controls early folliculogenesis via many cellular events like PGF2a and E2 receptor expansion, enhance P4 and E2 biosynthesis in granulosa cells of preovulatory follicle [9]. GDF9 preserve follicular structure and support growth of preantral follicles [10]. Hayashi et al. (1999) [11] observed that the use of GDF9 alone or in combination with FSH has a positive effect on mice secondary preantral follicles growth. Additionally, the daily growth increment of goat preantral follicles is related with GDF9 treatment, likewise, GDF9 treatment recorded increase in follicles diameter [12].

The Ovine *GDF9* gene is an autosomal, which codes the oocyte-derived GDF9, this factor is essential for normal folliculogenesis and ovulation [13, 14]. *GDF9* gene maps in chromosome 5 [13]. *GDF9* gene span about 2500 base pairs (bp), it contains two exons and single intron that interpose between the two exons, exon I spans 397 bp that encodes 1-134 amino acids, and exon II length about 968 bp that encodes 135-456 amino acids, while Intron I span 1126 bp [15].

The mice *GDF9* gene deletion (knockout) which, in turn, leads to complete sterility due to arresting of folliculogenesis at primary stage, and this confirm that the GDF-9 is essential for mouse follicular activation and growth [16, 17, 18, 19].

Numerous studies pointed out that GDF9 gene polymorphism play as genetic marker to improve the domestic animals reproduction; a point mutation in exon 2 of ovine GDF9 effect positively (improves fertility and litter size) [20]. As well as, the exon 1 mutations record same findings [21]. Likewise, several hereditary loci contributed to stimulate the breeding out of season in sheep; whereas many studies find a relationship between the polymorphisms in melatonin receptor 1A gene [22], aryl alkyl amine-N-acetyl-transferase gene [23] and deiodinase iodothyronine gene [24] with seasonality in different ewes breeds.

According to recent study, two breeding season were recorded in Iraqi awassi ewes, April, and autumn [25], that mean the non-seasonal awassi ewes breeding earlier (in April and May) than seasonal ewes (in autumn) at same circumstances. Although GDF-9 is an essential for, follicular, development, this study was performed to find a relationship between GDF9 mutation and breeding out of season in awassi sheep.

MATERIALS AND METHODS

Experimental animals and samples collection

Thirty non pregnant ewes with average age three years were used in this study in Baghdad province/ Iraq. This expermintal was approved by the Ethical Committee. The animals were disconnected into two categories depending on lambing and breeding season; seasonal and out of seasonal groups. At all time; animals were housed in one flock with a breeding ram in the Animal House. Blood samples (three ml per ewe) were collected randomly from 15 mature multiparous Awassi ewes along with data lambed in September and November/ 2018 (estus occurs at April and May/ 2018) (first group) and from 15 ewes lambed in mid December and January (estus occur at June and July) (second group) according to Hatif and Younis (2018) [23]; Younis et al (2019) [25]. The blood samples were aseptically aspirated from vena puncture of jugular vein into heparin sodium containing collection tube (APTACA/Italy) and stored at -20°C for DNA extraction, amplification and *GDF9* part gene (exon I) sequencing.

DNA extraction and *GDF9* amplification

Genomic DNA was isolated from stored blood samples of both groups via G-spin Kit (INtRON/ Korea) according to manufacturer's kit protocol. DNA quantity and purity of each sample were assessed by UV light and agarose gel electrophoresis. Two primers were designed to amplify Exon I of ovine *GDF9* according to Nanekarani et al. [26] (**Table 1**).

Table 1. Sequence of primers, melting temperature (Tm), and length of Exon I of *GDF9* gene

Primer	Sequence	Tm (°C)	GC (%)	bp
Forward	5-GAA GAC TGG TAT GGG GAA ATG-3	62	52.3	21
Reverse	5-CCA ATC TGC TCC TAC ACA CCT-3	62	52.3	21

Polymerase chain reactions were performed in a 25 µL reaction mixture containing approximately; genomic DNA (2 ul), of each primer (1 µl with concentration 10 pM), PCR Master Mix Kit (Intron/ Korea) (12.5 ul), and nuclease-free water (8.5 ul). The reaction program was described below (**Table 2**) according to Hafezian (2011) [27].

Table 2. The amplification conditions for primers of the Exon I of *GDF9* gene

No.	Stage	temperature	Time	No. Of Cycle
1	Initial denaturation	94°C	5 min	1
2	Denaturation	94°C	45 s	35
3	Annealing	58°C	40 s	
4	Extension	72°C	1 min	
5	Final extension	72°C	10 min	1

Electrophoresis is used to separated PCR products on 2 % agarose gel in 1× TBE buffer, stained with Red safe stain (INtRON/Korea), in parallel with a 100 bp DNA ladder (Kapa/USA), and UV light is used to visualized products.

Sequencing and genotyping

The PCR products (amplicon) were sequencing successfully via Macrogen Corporation/ Korea (Sanger sequencing method). Homology search was conducted by using BLAST option, which is available online in NCBI. SNPs were determined by using BioEdit program and NCBI.

Statistical analysis

The Statistical Analysis System- SAS (2012) [28] program was utilized to observe the effect of polymorphism in the parameters of present study; Chi-square test was utilized for significant comparison between percentages, while T test was used to significant compare between means.

RESULTS AND DISCUSSION

PCR amplification, sequencing and genetic variability

The exon I of Awassi *GDF9* gene amplification appeared uniform fragments with size 462 bp when electrophoresed in 1% agarose gel (**Figure 1**).

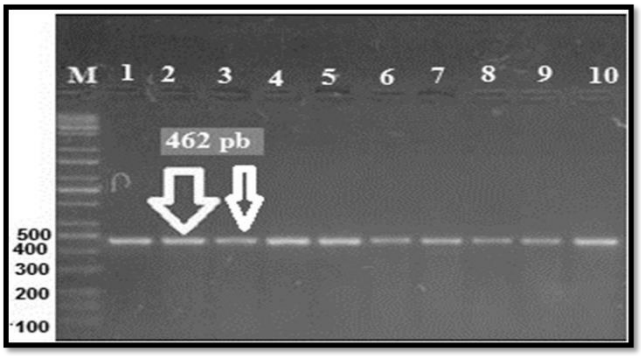

Figure 1. The PCR product of exon I *GDF-9* gene. M = DNA ladder 100–10000 bp. Lane 1–10 = PCR samples

The sequencing revealed one SNP G(129)R in chromosome 5 of *GDF9* gene (exon I) when compared to Santa Inês sheep breed *GDF9* gene, Sequence ID: FJ429111.1, without resulted in an amino acid (**Figure 2**) (**Table 3**).

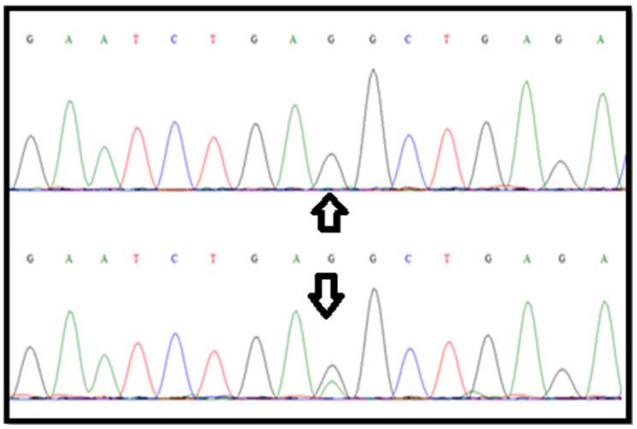

Figure 2. Wild-type and new variant G(129)R of exon I *GDF-9* gene

Table 3. Type of substitution of exon I *GDF-9* gene in Awassi ewes

No.	SNP location	Nucleotide change	Amino acid change	Type of mutation	Predicted effect
1	G(129)R	GAG > GAA & GAG	glutamic acid > glutamic acid (43)	Silent Mutation	Transition

Correlation between *GDF9* gene genotypes and breeding season, in awassi breed

The genotypic distributions of exon 1 *GDF9* gene in the two animal groups were recorded. The Outcomes demonstrated that the different *GDF9* genotypes could significantly affect (P<0.001) the seasonality in both groups. Higher significant variation (P<0.001) were recorded between the GG and GA genotypes of G(129)R locus. In seasonal Awassi ewes group; higher significantly increased (P<0.001) appeared in wild homozygote genotypes (GG) as compared with the mutant heterozygote genotypes (GA) of G(129)R locus. On the other hand, higher significantly increased (P<0.001) were recorded in wild genotypes (AA) of out of seasonal Awassi ewes group in correlation with other genotype (GG) of same locus. The genotypic frequencies and sequence polymorphisms of exon I Ovis aries *GDF9* gene in Awassi ewes showed in (**Table 4**).

Table 4. Genotypes structure frequencies of exon I of GDF-9 gene locus for Awassi ewes

Locus	Geno types	Observed Genotype	Genotypic frequency for seasonal ewes	Chi square	Observed Genotype	Genotypic frequency for non-seasonal ewes	Chi square
G(129)R	GG	19	63.00	9.29 **	12	40.00	7.90 **
	GA	11	37.00		18	60.00	

** (P<0.001)

By utilizing exon I of *GDF-9* gene amplification and sequencing analysis, the mutation (G > R) at coding base 129 was observed. The nucleotide variant G(129)R not cause amino acid (glutamic acid) changes at locus (43). According to genotyping method and by comparison, the phenotypic features with genotypes, the effect of SNP was predicted. According to table 4, highly signficant variations between genotypes and alleles frequencies of the two groups, genotypic frequency of GA was pre-eminent in non-seasonal ewes, while GG genotype was superior in seasonal ewes. This mutation was recorded previously in awassi ewes.

The finding of present study leads to speculate that the mutations maximize *GDF9* expression and caused increasing its function on ovarian follicle's growth and maintenance in ewes that breed off season. This suggestion since that the factor participates in most events that occur in the ovary, because this protein was expressed in cumulus cells, granulosa cell, as well as, in oocyte along oestrous cycle in cow, sheep, goat, pig and buffalo [9, 15, 29, 30), and bind to a type 1 receptor (TGFβR1) in the ovaries [2]. Many researches have been reported that the *GDF9* play a fundamental role in folliculogenesis and ovulation [13, 14].

The present study showed that non seasonal ewes possess the heterozygote GA genotype, and seasonal group that have homozygote GG genotype, that's mean estrus cycle stimulate earlier (2 to 3 months) in non-seasonal ewes than seasonal. Therefore, the polymorphism has a spur effect on growth and development of the follicles in non-seasonal ewes as a compared with seasonal ewes. The recent study was in agreement with Al-Mutar et al (2018) [21] finding, which showed that GA genotype of G(129)R locus was recorded significant increased (P<0.05) in percentage of follicles (4-8mm) and oocytes number as compared with wild GG genotype. Moreover, the recent investigation are in agreement with Hanrahan et al. (2004) [5], who infer that the SNPs in both exon I and II of ovine *GDF9* were related with enhance fertility and, ovulation, rate in, heterozygous, allele, for Belclare, and Cambridge, breeds.

The consequences of this investigation came consistently with the fact that the positive effect of GDF-9 on preantral, follicular, growth and ovulation. Almeida et al (2009) [12] found out that GDF-9 treatment to culture media accomplish antrum form to cultured follicles (62- 78%) as a compared to control (43.1%). Additionally, GDF9 control the granulosa cells expansion and differentiation in advanced follicles; also, stimulate basal, steroidogenesis, in granulosa cells [31]. Furthermore, GDF-9 is not only substantially for the commencement of early follicle development ,but also it contributes to follicular growth at later, stages, of follicle, growth, in mice [17]. Morever, intraovarian GDF-9 controlled the, transition, of the growing follicle from the pre-antrum, to early antrum, stage [32], and under the influence of gonadotropins;, the antrum, is formed, and the dominant, follicles is continued to grow until they come to the preovulatory, stage [33, 34].

As a conclusion, heterozygote *GDF9* gene polymorphisms of exon I (GA) of G129R was related with Awassi ewes that breeding out of season, while wild GG genotypic frequencies were higher in Seasonal Awassi ewes.

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