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Polymorphism of GDF9, BMP15, BMP1B, KISS-1 and GPR54 genes and their relationship with litter size in two Egyptian sheep breeds with expression analysis of KISS-1 and GPR54 genes

Nermin El-Halwany[1], Abdel-Aziz K.B[1], Sherif Mourad[2], and Ahmed F.M. Al-Tohamy[1]*.

[1] Department of Cell Biology, Genetic Engineering and Biotechnology Research Division, National Research Centre, Dokki, Giza, Egypt.
[2] Department of Chemistry, Faculty of Science, Cairo University, Egypt.

ABSTRACT

Reproductive performance is an important economic trait in sheep. This study was carried out to investigate the effect of some polymorphic sites, that assumed to be linked to fertility and reproduction process, on Egyptian sheep prolificacy. In the first part, 12 SNPs that locate in 5 different genes (GDF9, BMP15, BMPR-1B, KISS-1 and GPR54) were genotyped using the iPLEX Gold technology (SEQUENOM) in both Rahmani and Ossimi ewes. Only one mutation showed polymorphism that is a 5 bp insertion / deletion (TTCTT) in 5´ regulatory region of the GPR54 gene. However, no significant association was observed between this polymorphism and litter size. In the second part, two genes (KISS-1 and GPR54) were subjected for gene expression analysis using real time PCR technique in different ovarian cell compartments. The results showed that both KISS-1 and GPR54 genes were expressed in both mature and immature oocytes. However, the two genes were strongly expressed in bad quality oocytes compared to good quality oocytes. GPR54 gene was expressed in granulosa cells and corpus leuteum tissue while KISS-1 gene was neither detected in granulosa cells nor corpus leuteum tissue. The results of gene expression could be used for better understanding of ovulation process in sheep.

Keywords: SNP, Sheep, litter size, polymorphism, Gene expression, prolificacy.

Corresponding author

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INTRODUCTION

Sheep is considered as one of the first livestock to be domesticated by humans. It lives in a widespread range of environmental circumstances, as a result of its flexibility to survive with little nutrients and controllable size [1]. That resulted in vast morphological variation among different populations [2,3]. Sheep are used for their meat, milk and wool production which were exploited since domestication [3,4].

In Egypt, a lot of work was done to enhance sheep production systems through crossing native Egyptian breeds with introduced breeds. However, these introduced breeds require costly considerations, such as climate controlled housing, regular doses of antibiotics, and excessive amounts of high protein feed. Therefore, the development and conservation of locally adapted breeds in developing countries including Egypt are crucial for continued sustainable exploitation of these higher stresses, medium and low input production systems. Hence, it is more rational to direct the breeding strategy to improve productivity of the local breeds in terms of producing a higher number of lambs, meat and milk. The number of live lambs born per breeding ewe-litter size -is an important trait in sheep breeding programs. As more sheep would be translated into more products, which would lead to higher income.

Using the molecular tools has helped in the identification of polymorphisms associated with phenotypic variation of complex traits [5]. In this context, information about genetic factors affecting prolificacy would increase the selection accuracy in sheep breeding programs. During last decades, mutations in many genes have been reported to affect ovulation rate in different sheep breeds, these genes include: GDF9 (FecGH), BMP15 (FecXG, FecXB, FecXI, FecXH, FecXL, FecXR), BMPR1B (FecB or booroola) as reviewed in Qiuyue et al. (2014) [6]. More recently, other genes associated with sheep fecundity have been identified such as, prolactin receptor gene, estrogen receptor gene, POU1F1 gene, FSH receptor gene, INH gene and KISS-1 gene [7]. Polymorphisms of the KISS-1 and GPR54 genes were reported to be linked to prolificacy and litter size in sheep and goats [8]. The current study concerned with investigating the Egyptian Rahmani and Ossimi breeds for the presence of 12 SNPs that locate in 5 different genes (GDF9, BMP15, BMPR1B, KISS-1and GPR54). Also to characterize the dynamic expression patterns of two genes (KISS-1, GPR54) in immature and mature oocytes, granulosa cells and corpus luteal tissue.

MATERIALS AND METHODS

Animal population and blood collection:

A total of 76 Egyptian ewes were used; including 32 Ossimi ewes and 44 Rahmani ewes. Those animals were born between 2002 and 2010. The blood samples were collected under supervision of local veterinarians under sterile conditions. The two sheep breeds were reared in the research farms belong to Animal Production Research Institute, Giza, Egypt under the same nutritional and environmental conditions. Parity number, birth weight of lamb and mother age was recorded for each ewe.

Genomic DNA extraction:

Genomic DNA was isolated using commercial kit (GF-1 vivants) according to the manufacturer's instruction. The DNA quantity was determined using spectrophotometer. The quality of DNA samples was checked on 1.5 % agarose gel electrophoresis. The samples of good quality were used for genotyping.

SNP Genotyping using iPLEX MassARRY MALDI-TOF MS:

A total of 12 SNPs located in 5 genes (GDF9, BMP15, BMPR1B, KISS-1 and GPR54) were selected to be genotyped in the examined animals using Sequenom custom panel on the Sequenom MassARRAY platform by Neogen genotyping service (USA, www.neogen.com). The selected SNPs and their positions are summarized in table (1).

Association Analysis

Observed litter size of the ewes was tested for association with the detected SNP according to the following model using GLM option in SAS, version 9.4 (SAS 2014, SAS Institute Inc., Cary, NC, USA).

$$Yijklmn = \mu + Bi + Pj + YSk + Agel + Gm + Eijklmn$$

where Yijklmn is a litter size at birth; μ is the overall mean of observations; Bi is the fixed effect of breed; Pj is the fixed effect of parity order for each ewe; YSk is the fixed combined effect of year (y) and season (s) of lambing; Agel is the fixed combined effect of age at first lambing; Gm is the effect of INDEL genotypes and Eijklmn is the residual error. Post hoc, effects among INDEL means were tested for significance using Duncan test as implemented in SAS (SAS 2014, SAS Institute Inc., Cary, NC, USA). Frequency estimations of the INDEL genotypes were calculated by direct counting.

Table (1) The selected SNPs in different candidate genes and their positions.

SNP ID	Gene name	Chromosome	Chromosomal position	Breed	Reference
SNP1	GDF9	5	41841212	Bel/Cam	[15]
SNP2	GDF9	5	41841285	Thoka	[16]
SNP3	GDF9	5	41841362	Santa Ines	[12]
SNP4	BMP15	X	50971111	Hanna	[17]
SNP5	BMP15	X	50971170	Inverdale	[17]
SNP6	BMP15	X	50971224	Bel/Cam	[15]
SNP7	BMP15	X	50971249	Bel/Cam	[15]
SNP8	BMP15	X	50971460	Lacaune	[18]
SNP9	BMPR1B	6	29382188	Booroola	[14]
SNP10	KISS-1	12	1303482	Jining Grey goat	[34]
SNP11	KISS-1	12	1305155	Jining Grey goat	[34]
SNP12	GPR54	5	40843495	Small Tail Han	[40]

Oocyte collection & maturation

Sheep ovaries were collected from local slaughter-house and transported to the lab within 2 hours. The corpus luteal tissues were excised from the ovaries and kept at -80ºC until RNA extraction. The cumulus oocyte complex (COC) were aspirated from antral follicles of 2-8 mm in diameter using a 20-gauge (20-G) 1-inch needle and a sterile disposable 10 ml syringe containing approximately 0.2 ml of collection media that consists of TCM-199 (Sigma, St. Louis, MO), 2% heat inactivated fetal bovine serum (FBS; Gibco, Gaithersburg, MD), heparin (Sigma), and penicillin/streptomycin [9]. Aspirated oocytes were transferred to a petri dish with fresh collection media without heparin. Then, oocytes were evaluated based on morphology and categorized as good or bad grades according to Thompson et al. (1995) [10].

All oocytes were washed three times in maturation media (TCM-199, 10% FBS, ovine FSH [oFSH-RP-1; NIAMDD-NIH, Bethesda, MD], ovine LH [oLH-26; NIADDK-NIH], estradiol [Sigma], glutamine [Sigma], sodium pyruvate [Sigma], and penicillin/streptomycin [9]. Half of the good quality COCs were incubated in stabilized (matured for 21-24 hours under oil at 39º C, 5% CO_2, and 95% air) maturation media.

For the remaining COCs, the cumulus masses were mechanically separated from the oocytes by repeated pipetting. The resultant granulosa cell suspension was washed in culture medium, collected by centrifugation and kept at -80ºC for RNA isolation. Similarly, after oocyte maturation, the cumulus cells were removed as previous, and kept for RNA isolation. Both immature and mature denuded oocytes were washed once with PBS and treated with tyrode's solution for 20s to remove the zona pellucida and snaped-frozen in a cryotube containing Igepal lysis buffer (Sigma), 40Ul-1 Rnasin (Promega) and 5mM dithiothreitol (Promega) until use for RNA isolation. So we had 5 pools of different ovarian cells and 3 different corpus luteal tissues to be examined for the expression levels of KISS-1 and GPR54 genes.

Real Time Polymerase chain reaction (RT-PCR)

Messenger RNAs were isolated from the following pools: Mature Good Oocyte (Mat G), Mature Bad Oocyte (Mat B), Immature Bad Oocyte (Imm B), Immature Good Oocyte (Imm G), Granulosa Cells (Gran) And Corpus Luteal Tissue (CL) using oligo $(dt)_{25}$ attached magnetic beads (Dynal) according to manufacturer's instructions. Total RNA was isolated from corpus luteal tissue (from 3 different animals) using (Promega, USA) kit following manufacturer's instruction.

Isolated mRNAs were reverse transcribed into cDNA using oligo dT (11) primer with RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. Each PCR run was performed in duplicate to control the reproducibility of the quantitative results.

Quantitative real time PCR analysis was conducted on the examined ovarian compartments. The appropriate concentrations of primers were added to reaction mixture that contained 10 µl of SybrGreen Supermix (Invitrogen), 1 µl of cDNA, with the remainder nuclease free water to a final volume of 20µL. Real-time PCR amplification was conducted with the following conditions: initial denaturation at 95º C for 5 min, followed by 40 cycles of denaturation at 94º C for 30 S, annealing at 55º C for 1 min, and extension at 70º C for 40 s. Final extension at 72º for 3 s.

Following PCR amplification, a melting curve analysis was conducted between 55ºC and 95ºC in 0.5ºC increments every 10s. The data were analyzed by the comparative threshold cycle (ΔCt) method and the mean input amount of each gene was normalized to the mean input amount of B-actin as an endogenous internal standard. The mRNA levels were reported relative to mature good oocyte as a calibrator (control).

RESULTS

The genotyping results of the examined 12 SNPs in 5 different genes (GDF9, BMP15, BMPR-1B, KISS-1 and GPR54) are summarized in table (2). The results showed that, only one polymorphism which is a deletion of TTCTT in GPR54 gene was detected in both Rahmani and Ossimi breed. However, this polymorphism showed no significant association with the observed litter size as illustrated in table (3)

Table (2) Genotyping results The selected SNPs in different candidate genes

SNP ID	Gene	Chr.	Nucleotide Change	Polymorphism
SNP1	GDF9	5	C>T	Monomorphic
SNP2	GDF9	5	A>C	Monomorphic
SNP3	GDF9	5	T>G	Monomorphic
SNP4	BMP15	X	C>T	Monomorphic
SNP5	BMP15	X	T>A	Monomorphic
SNP6	BMP15	X	C>T	Monomorphic
SNP7	BMP15	X	G>T	Monomorphic
SNP8	BMP15	X	G>A	Monomorphic
SNP9	BMPR1B	6	A>G	Monomorphic
SNP10	KISS-1	12	INS (TATGAGTAGCCCACCTGC)	Monomorphic
SNP11	KISS-1	12	G>C	Monomorphic
SNP12	GPR54	5	TTCTT / -----	Polymorphic

Table (3) Genotype frequency and association analysis of the polymorphism in GPR54 gene with litter size in Egyptian ewes

Genotype	Frequency	Mean ± SE	P-value
---/---	0.01	1.00±0.00	0.5216
---/TTCTT	0.25	1.26±0.41	
TTCTT/TTCTT	0.74	1.20±0.37	

Real-time PCR was used to investigate the mRNA levels of both KISS-1 and GPR54 genes in different ovarian samples (immature and mature oocyte, granulosa cells and corpus luteal tissue). The results showed that both genes were expressed in oocytes cells (mature and immature). However, the two genes were strongly expressed in bad quality oocytes compared to good ones. GPR54 gene was expressed in granulose cells and corpus leuteum tissue while KISS-1 gene was neither detected in granulose cells nor corpus leuteum tissue as shown in Figure (1).

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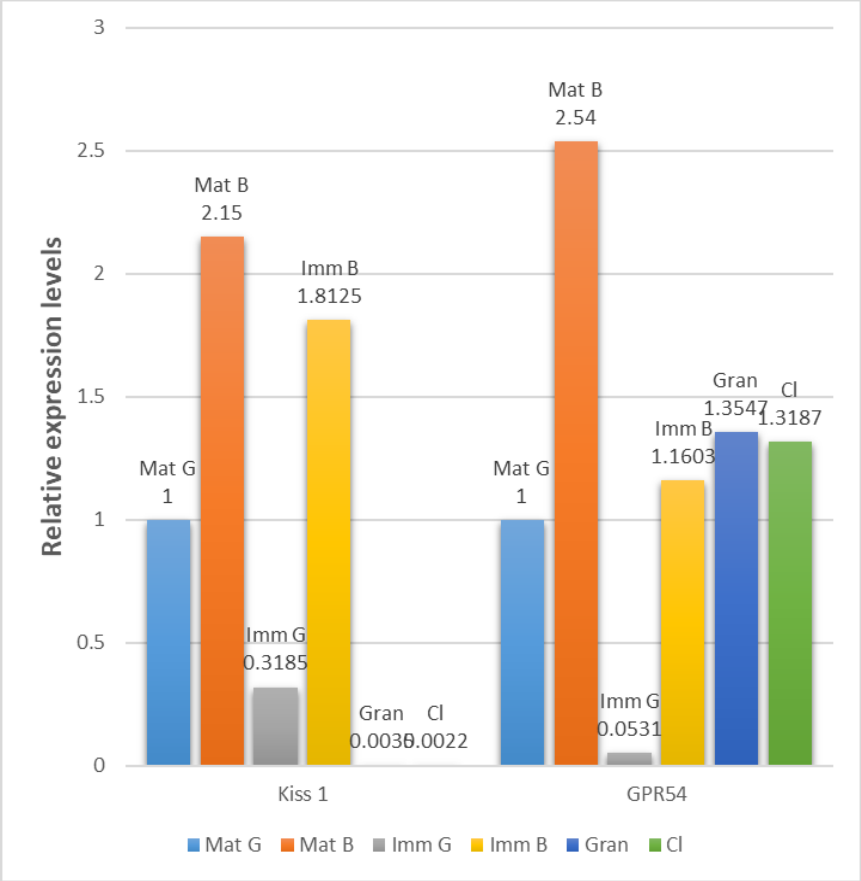
Figure (1) The expression Pattern of KISS-1 and GPR54 genes in different ovarian cell types



Mature Good Oocyte (Mat G), Mature Bad Oocyte (Mat B), Immature Bad Oocyte (Imm B), Immature Good Oocyte (Imm G), Granulosa Cells (Gran) And Corpus Luteal Tissue (CL).

DISCUSSION

Litter size in sheep is determined mainly by the number of ovulations per estrous cycle (ovulation rate, OR). Several polymorphisms have been identified to be associated with an increase in both OR and LS. The majority of these polymorphisms are located in genes of the transforming growth factor beta (TGFb) superfamily or its receptor [11]. These polymorphisms are most prevalent in the bone morphogenetic protein 15 gene (BMP15) followed by the growth differentiation factor 9 (GDF9) and bone morphogenetic protein receptor type 1B (BMPR1b) genes [11]. Recently, KISS-1 and its receptor GPR54 genes were believed to play a major rule in reproduction process in sheep and may affect litter size [8]. In the current study, the Egyptian Rahmani and Ossimi breeds were examined for the presence of 12 SNPs that locate in GDF9, BMP15, BMPR1B, KISS-1 and GPR54 genes.

Previous studies reported several mutations in GDF9 like Embrapa [12], G7 [13] and recently Vacaria (FecGv) [14]. Those mutations, have an additive effect on ovulation rate and litter size, whereas Hanrahan et al. (2004) [15] and Nicol et al. (2009) [16] reported another mutation cause increased prolificacy in the heterozygous and infertility in the homozygous ewes.

Several mutations on BMP15 were demonstrated previously to be affect litter size and ovulation rate in sheep, Cambridge [15], Hanna, Inverdale [17], Lacaune [18], Belclare [15], Grivette and Olkuska [19] mutations. Also, a deletion of 17 bp in the breed Rasa Aragonesa. This deletion, located at the beginning sequence of exon 2 [20,21] leads to increase prolificacy in the heterozygous and infertility in the homozygous ewes.

In the current study, the Egyptian Rahmani and Ossimi breeds were examined for the presence of 3 SNPs in GDF9 that were described by Silva et al. (2011) [12], Hanrahan et al. (2004) [15] and Nicol et al. (2009) [16]. Also, 5 SNPs in BMP15 gene that were described by Souza et al. (2014) [14], Hanrahan et al. (2004) [15], Galloway et al. (2000), [17] and Bodin et al. (2007) [18]. However, non-of the examined mutations on neither GDF9 nor BMP15 were detected in both Rahmani and Ossimi Egyptian breeds. That could be due to differences between different breeds.

BMPR1B belongs to the family of receptors for transforming growth factor- (TGF). It is one of the specific type I receptors for the subfamily of BMP and GDF ligands [22]. Ligand binding induces the formation of heterotetramers of type I and type II receptors, leading to the activation of a specific intracellular signaling pathway by phosphorylation of Smad proteins [23, 24]. In sheep, it was found that BMPR1B ligands, GDF-5 and BMP-4, inhibit progesterone secretion by ovine granulosa cells isolated from small antral follicles (1–3 mm diameter) in vitro [25].

One-point mutation in the highly conserved intracellular kinase signaling domain of the BMPRIB caused an amino acid change (249Q>R) was associated fully with the hyperprolific phenotype of Booroola ewes [14,25,26]. In the present study this mutation showed polymorphic patterns in both Rahmani and Ossimi breeds. That could be due to small sample size and/or differences between different breeds. This result supports the previous study by El-Hanafy et al. (2009) [27] who examined this SNP using RFLP PCR in 5 Egyptian breeds (Rahmani, Ossimi, Awassi, Barki and Awassi x Barki crossbred), but he couldn't detect this SNP in the examined breeds.

KISS-1 gene was initially recognized in melanoma – a type of skin cancer- by Lee et al. (1996) [28] in experimentations intended to detect the molecules in charge of the anti-metastatic effect. That discovery strongly suggests that KISS-1 expression might suppress the metastatic ability of malignant melanoma cells [28]. GPR54 (or KISS-1 receptor) coupled to kisspeptins, has been discovered to perform an essential part for the initiation of puberty and also to suppress cancer metastasis [29,30]. The KISS-1-GPR54 pathway was observed to be engaged in adjusting reproductive function in mammals, principally in the initiation of puberty and sexual maturation by regulating the release of gonadotrophin-releasing hormone (GnRH) from hypothalamic neurons [28].

Mutations within KISS-1 and/or GPR54 are linked to hypogonadotropic hypogonadism -gonadotropin-releasing hormone deficiency- in humans [29,31], a phenotype which is also detected in mice [32]. In KISS-1 gene, 8 SNPs were initially detected in Chinese Han girls diagnosed with central precocious puberty (CPP) [33]. Cao et al. (2010) [34] detected 6 mutations in KISS-1 gene in 5 goat breeds with different prolificacy.

On the other hand, GPR54 gene displayed more polymorphisms; where several SNPs were identified in idiopathic hypogonadotropic hypogonadism [31,35,36]- and central precocious puberty (CPP) patients [37]. Li et al. (2008) [28] identified 7 mutations of GPR54 gene in White Duroc × Erhualian intercross pigs. Feng et al. (2009) [39] located 3 nucleotide mutations in goat GPR54 gene.

Up to our knowledge the literatures regarding the effect of KISS-1 and GPR54 genes on sheep reproduction are infrequent. Recently, some studies were performed to find potential association between KISS-1, GPR54 and litter size in sheep [8,40]. In the current study we investigated 2 SNPs in KISS-1 gene that were previously identified by Cao et al. (2010) [34] in Jining Grey goats, and 1 mutation in GPR54 gene which is a 5 bp deletion/insertion (TTCTT) located at the 163–167 in 5´-regulatory region. This mutation was previously identified by Tang et al. (2012) [40] in Tail Han sheep.

Genotyping results showed the absence of the 2 candidate SNPs of KISS-1 gene in both Rahmani and Ossimi breeds. That could be due to differences between different animals, as those 2 SNPs were identified in goats [34]. On the other hand, the deletion of TTCTT in GPR54 gene was detected in both Rahmani and Ossimi Egyptian sheep breeds. However, no significant associations were found between this mutation and litter size., This could be due to the small sample size. We recommend that this mutation should be investigated on a larger sample size to explore its effect on Egyptian sheep prolificacy.

In sheep, KISS-1 mRNA expressing cells are found in the arcuate nucleus and dorsolateral preoptic area. Both of them appear to facilitate the positive feedback influence of estradiol to generate the

preovulatory GnRH/LH flow [41]. The LH flow has been linked with an increase in the LH response to kisspeptin in humans and sheep [42,43], indicating that, the flow may be generated by increased kisspeptin output and sensitivity. In addition to KISS-1 and GPR54 prominent expression at hypothalamic levels, some evidences propose that KISS-1 and GPR54 are as well expressed in several peripheral reproductive tissues such as the ovary [44,45], oviduct [46] and testes [47].

In the current study, we investigated the mRNA levels of KISS-1 and GPR54 genes in different ovarian compartments including oocyte (immature and mature), granulosa cells and corpus luteal tissue using real-time PCR. Both KISS-1 and GPR54 genes were expressed in both immature and mature oocytes cells which points to the importance of these genes in oocyte ovulation. However, the two genes were strongly expressed in bad quality oocytes compared to good quality oocytes. Both KISS-1 and GPR54 genes where assumed to have an anti-metastatic effect [28]. Based upon that, their higher level in bad quality oocytes compared to good ones in the present study could suggest an apoptotic role of these genes in bad quality oocytes. However, further study is required to investigate this suggestion.

CONCLUSION

We investigated 12 SNPs that are located in 5 different genes (GDF9, BMP15, BMPR-1B, KISS-1 and GPR54). Genotyping of these SNPs in Egyptian Rahmani and Ossimi sheep breeds showed that only one polymorphic site was detected that is a deletion of TTCTT in GPR54 gene. No significant associations between the detected mutation and litter size were found. The mRNA expression analyses of KISS-1 and GPR54 genes have provided information that may shade light on role of these genes in sheep prolificacy and fecundity. Our recommendations for future studies include; increasing the sample size of the investigated animals as well as sequence analysis of the whole KISS-1 and GPR54 genes of Egyptian breeds to screen them for potential SNPs in attempt to discover novel genetic markers.

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