IJAS Iranian Journal of Applied Animal Science

Search for Association between Ovine Wingless-Type MMTV Integration Site Family Member (*Wnt10A*) Genes with Supernumerary Teat in Ghezel and Romanov Sheep

Research Article

S. Eydivandi[1], S. Eghbalsaied[2], M. Momen[3], E. Khajouei[4], A. Javanmard[5*] and S.M. Ghoreishifar[6]

[1] Center for Quantitative Genetics and Genomics, Blichers Allé 20, 8830 Tjele, Denmark
[2] Transgenesis Center of Excellence, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran
[3] Department of Animal Science, Faculty of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran
[4] Department of Animal Breeding and Genomics Center, Wageningen University and Research, the Netherland
[5] Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
[6] Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

Received on: 12 Mar 2021
Revised on: 1 Sep 2021
Accepted on: 16 Sep 2021
Online Published on: Jun 2022

*Correspondence E-mail: a.javanmard@tabrizu.ac.ir
© 2010 Copyright by Islamic Azad University, Rasht Branch, Rasht, Iran
Online version is available on: www.ijas.ir

ABSTRACT

Supernumerary teats (SNT, polythelia) is a common abnormality in many mammalian species including sheep. Recently, the *WNT10A* candidate gene has been reported to be associated with regulation of embryonic programming for mammary gland connective tissue differentiation and embryogenesis. Here, we propose that variants of this candidate gene may be linked with SNT in Ghezel (GHE) and Romanov (ROM) sheep. Therefore, a case-control study was conducted to uncover this abnormal phenotype. The study group consisted of fifteen individuals which had four functional teats (GHE=10, ROM=5). The control group consisted of thirty individuals which each had two (normal) teats (GHE=20, ROM=10). After extraction of genomic DNA from all samples, the candidate gene region was amplified using routine PCR. Genotyping at the *WNT10A* gene was performed by sequencing of the purified amplicons. Genotypes and allele frequencies were compared between the two study groups using the χ^2 statistical test. A P-value of 0.05 was considered to be the threshold below which all results were significant. No effect of allelic variation (A/G in exon 4) between case and control groups in GHE and ROM sheep was found. In summary, our study provides preliminary results linking candidate gene this single nucleotide polymorphism to SNT in GHE and ROM sheep.

KEY WORDS mammary gland, sheep, single nucleotide polymorphism, supernumerary teats, WNT signaling.

INTRODUCTION

Supernumerary teats (SNT, also called polythelia) is a common abnormality in many mammals, including sheep. Normally, the ovine mammary gland develops two functional teats to nurse the lambs during the pre-weaning period. The occurrence of SNT is viewed as an undesirable characteristic that can negatively affect milk production, as well as causing a longer milking period and udder injuries (Kenny, 2014). Orphan lambs are the negative consequence of large litter sizes in sheep, and therefore, producing sheep breeds with four functional teats may be one possible strategy to overcome the problem with orphan lambs. Although the surplus teats in SNT can be completely functional in regards to milk production, these teats are usually non-functional and have a greater risk to become infected with mastitis-causing pathogens (4.3% in sheep, and 29.7% in goat) (Kenny, 2014). In addition, the cause of SNT is prob-

ably due to abnormal prenatal hormones and the intrauterine position of the fetus (Brka *et al.* 2002). Currently, little attention has been paid to the genetic prevalence of SNT in ewes with four healthy and functional teats. In particular, assuming two teats are beneficial in terms of fecundity, four teats might be more efficient for suckling lambs. Theoretically, a sheep with four teats should be able to raise twice as many lambs than a two-teats sheep thus cutting the production costs in half.

A series of recent studies have indicated that SNT is ascribed to certain breeds in various mammalian species and geographical area. These studies mostly were done on mice, pigs, humans, buffalo, cattle, goats and sheep (Vainikainen, 1945; Brka *et al.* 2002). In sheep and goats, this trait is linked to prolificacy (Kenny, 2014), and considered as a desirable trait (Lundeheim *et al.* 2013). Interestingly, the genetic background of supernumerary teats has received the attention of researchers in the past decade. For example, Kenny (2014) provided evidence for low to medium heritability of SNT, with a frequency of 20-60 % in Finnish Landrance and Coopworth ewes. They also reported that inbreeding and selection for litter size can increase the probability of its occurrence in a particular breed. However, in order to understand the genetic background of SNT, we need to take a look at the DNA and search for potential mutations in candidate genes.

There are several studies addressing various candidate genes responsible for supernumerary teats. Fibroblast growth factor (FGF), T-box transcription factor, parathyroid hormone-related protein (PTHrP) and wingless-related integration site (WNT) have been reported so far as necessary factors for formation and maintenance of the mammary bud (Kenny, 2014).

Moreover, using both quantitative trait loci (QTL) analysis and the direct candidate gene approach, the member WNT10A of the WNT family was reported as being a candidate gene for abnormal teat development (Pausch *et al.* 2012). Generally, the *WNT* gene family is important for cell fate decisions, proliferation, adhesion, cell shape and cell movements, and plays a crucial role in early embryonic development. It is highly associated with numerous cancers as its members can act as oncogenes or tumor suppressor genes (Abedini *et al.* 2015). The WNT gene is located on chromosome 2 of the sheep genome, consisting of 4 exons which are translated into a protein comprised of 416 amino acids.

Based on the reported lines of evidence, we designed the current study to search for a potential causal polymorphism within the ovine wingless-type MMTV integration site family member *WNT10A* gene in GHE and ROM sheep, having either two or four functional teats to fill this literature gap.

MATERIALS AND METHODS

Animals and sampling

We approached most North West regional sheep holders of Iran to ask for collaboration in this case study by providing us with sheep for sampling. The case group consisted of fifteen individuals, each having four functional teats, from both breeds (GHE=10, ROM=5) (Table 1). The control group consisted of thirty individuals, each having two normal teats, again from both breeds (GHE=20, ROM=10).

Figures 1 and 2 show Ghezel and Romanov sheep with SNT i.e. four functional teats and normal milk secretion during feeding time.

Table 1 An overview of the experimental case-control individuals in both sheep breeds

Breed	n	Category	n
Ghazel	15	Control	20
		Case	10
Romanov	30	Control	10
		Case	5

Blood collection, genomic DNA extraction and PCR

We collected blood from the jugular vein using vacuum tubes coated with K_2-EDTA anticoagulant. The collected blood samples were immediately transferred onto ice, moved to the laboratory, and kept in a -20 ˚C freezer. Next, genomic DNA was extracted using a commercial DNA purification kit (Roje, Yazd, Iran). DNA quality and quantity were checked using horizontal electrophoresis with low melting agarose and ethidium bromide (0.5 g/mL) staining.

PCR amplification and sequencing at the WNT10A locus

In our preliminary experiments, a 346 bp fragment was amplified from the WNT10A locus with the following primers: forward: 5´-TCTGCCCCTCTCTATGTCC-3´ and reverse: 5´-GGAACTCCGTGGCTCAGG-3´, corresponding to the accession number: XM_004004935.4. The polymerase chain reaction (PCR) reaction included 25 µL total volume comprising of 0.1 mM of dNTP, 2.5 mM of $MgCl_2$, 0.5 mM of each specific primer, PCR Buffer (1X), 2 U of Taq polymerase (Denmark) and 50 ng/µL of DNA template. PCR amplifications were performed with an initial denaturation step (94 ˚C for 5 min), followed by 32 cycles at 94 ˚C for 60 s, 72 ˚C for 60 s, and 72 ˚C for 5 min. Horizontal gel electrophoresis was done for 40 min in a 2.5% low melting agarose gel, then stained with Red safe dye and visualized under UV light. A PAC1000 power supplier (Bio-Rad, USA) was used during electrophoresis. A standard size marker ladder in increments of 100 bp was applied to estimate the size of the PCR products (fermentas) using computer software BIO 1D++.

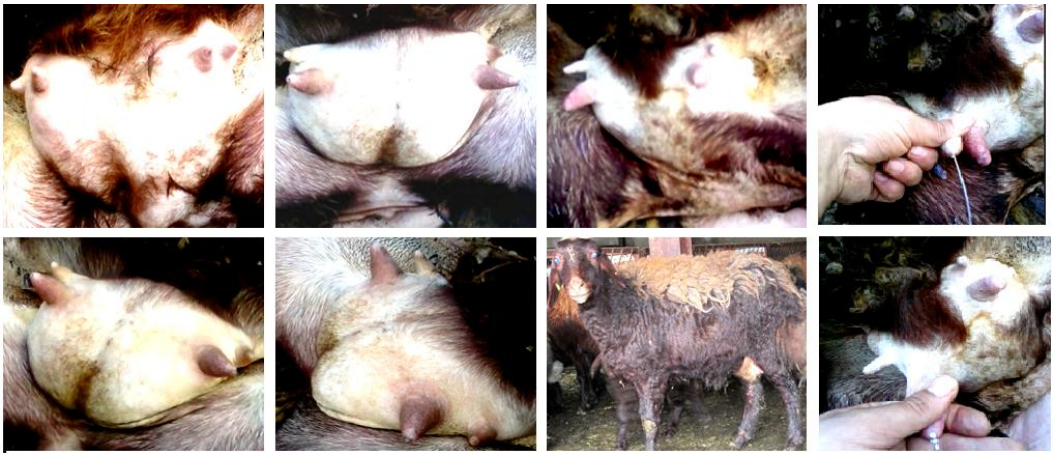

Figure 1 Supernumerary teat (polythelia) in Ghezel ewes with four functional teat having a normal milk secretion

Figure 2 Supernumerary teat (polythelia) in Romanov sheep ewes with four functional teat

Purified PCR products of the targeted size were submitted to the Sanger sequencing service (Macrogen DNA Sequencing Service, Korea). Sequence alignment was performed using the online tools BLAST, MAFFT and CLUSTW (www.ebi.ac.uk).

Statistical analysis

After sequencing the target genome segments, we did a pairwise BLAST analysis of the whole target sequence against the gene bank database sequences. So, the similarity and identity indices (validity of sequencing of targeted gene) were computed using MAFFT online software (https://mafft.cbrc.jp/alignment/server/cgi-bin/mafft5.cgi). Then, number of N (non red based) and Phred quality control index (grey bar) were monitored corresponding to each colored peak. Sequenced nucleotides that showed higher than a 20 QC score were considered as genuine sequences. Comparison of allelic and genotypic frequencies between the case and control groups were carried out using the Chi-square test, considering a p-value below 0.05 as significant.

KEGG pathway, PPI Interaction network and generation of the 3D protein modeling

We searched for *WNT10A* gene biological and cellular function using bioinformatics view using KEGG and additionally Cytoscap based analysis for predict protein–protein interactions networks and for generation of the three-dimensional model of *WNT10A* gene proteins SWISS-MODEL Repository was applied.

RESULTS AND DISCUSSION

Our study shows that the frequency of four healthy teats significantly differed between the two breeds, and ranged from 2 to 10% in the GHE and ROM sheep, respectively. The ROM breed is well-known for giving birth to multiple twins. Therefore, the main commercial advantage of ewes with four functional teats is their ability to rear more lambs successfully by allowing all to suckle at the same time thus reducing shepherding time and labor costs in flocks with high lambing rates.

Figure 3 illustrated observed frequency of four functional teats in this study.

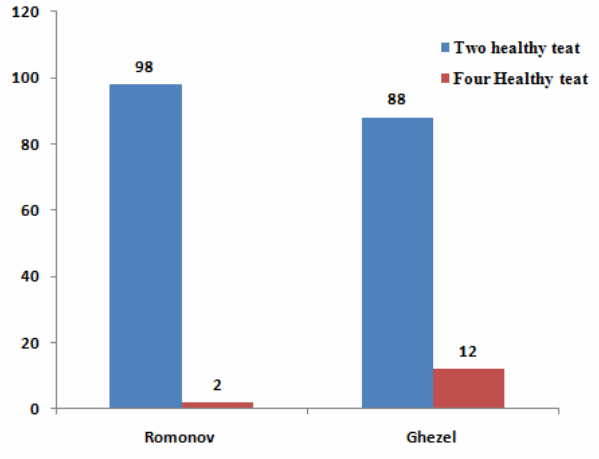

Figure 3 Observed frequency of four functional teat in this study

Figure 4 shows the PCR product of the WNT10A gene with the expected size of 346 bp, and in sufficient amount suitable for sequencing (Figure 3).

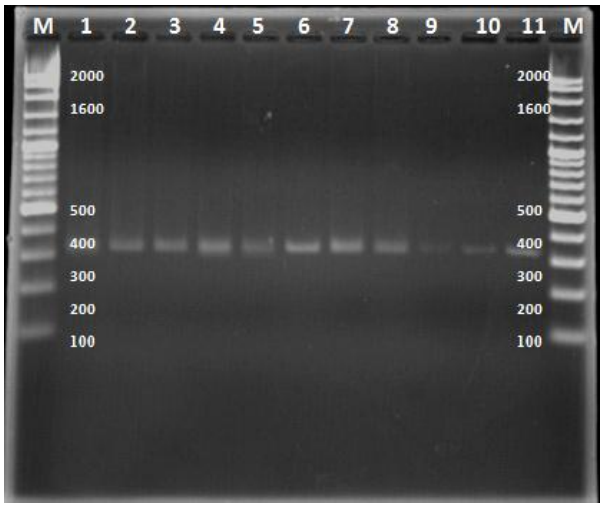

Figure 4 The quality of amplification of 345 bp PCR product of candidate gene in the present study

From the sequencing data in Figures 5 and 6, the case-control category mostly showed the presence of Adenine base (A) in the position of 185 bp of amplicon in both breeds, therefore, eliminated for further statistical association study. Table 2 summarizes the results obtained from analysis of the sequenced segment in the *WNT10A* gene, in two breeds.

Here, our study showed that the frequency of four healthy teats obviously is different among the investigated breeds and had a range from 2% in ROM to 10% in GHE, respectively. A logical explanation why sometimes breeders avoid driving to large litter size in sheep may relate to problematic issues of triplet births: smaller, weaker lambs for their competitions for limited sucking milk source and teat number.

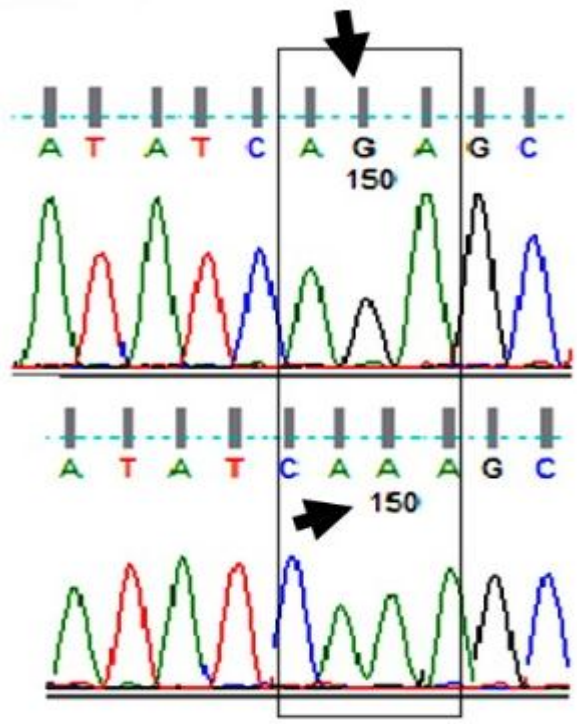

Figure 5 Validation of A/G polymorphism in 345 bp segment of WNT10A gene

Research on SNT has a long history, SNT can be functional (i.e., with the emission of milk) or nonfunctional, though SNT teats are not restricted to females and can be observed in males (Kenny, 2014). SNT was detected in approximately 17% of Turkish Saanen goats of a herd (Martin *et al.* 2017). In addition, 1.65% of the goat population examined in Bihar, India, possessed SNT (Ozoje, 2002). Furthermore, it has been reported the occurrence of SNT is around 44% of the goat population. In a survey on 589 West African Dwarf goats (Bemji and Popoola, 2011), an incidence of 7.3% of SNT was reported. On the other hand, several studies have reported that the prevalence of SNT is a primary udder abnormality in both WAD and RS goats indigenous breeds in Nigeria (Amao *et al.* 2003).

Similar studies on SNT (Oseni *et al.* 2006) and Adebayo and Chineke (2011) revealed that 64.3% and 5% of goats in the southern and South Western part of Nigeria possess extra teats, respectively. In this study, we investigated the pattern and variability of functional teats and their association with the candidate gene approach in GHE and ROM (carrying two normal teats versus the unexpected four healthy teats).

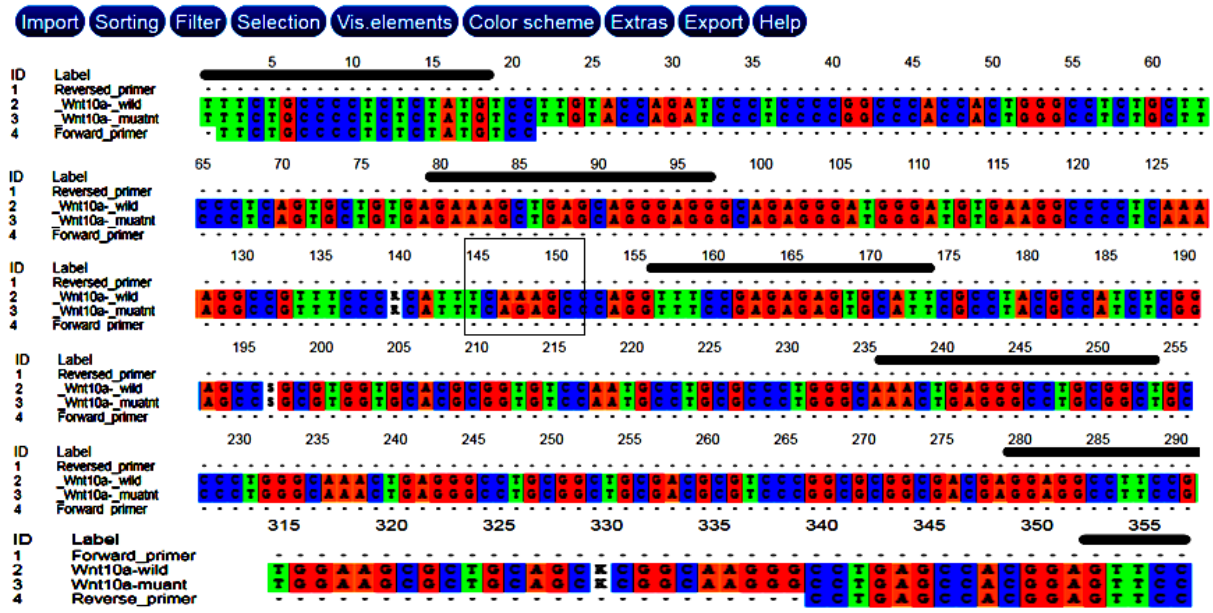

Figure 6 Outputs of alignments for sequencing raw data of *WNT10A* gene in this study

Table 2 Summary of sequencing results for *WNT10A* gene in two investigated breeds

Breed	Number of samples	Category	Number of samples	Adenine	Guanine
Gahzel	15	Control	5	4	1
		Case	5	5	0
Romanov	30	Control	5	5	0
		Case	5	5	0

Theoretically, we expected crossing four-teat rams with four-teat ewes, may produce offspring with necessarily functional four nipples. Also, we hypothesized that the occurrence of mutation in the *WNT10A* candidate gene could be responsible for increasing the teat number in sheep species. Generally, the results of PCR-sequencing in the *WNT10A* gene generated the same patterns and were in the accordance with earlier reports.

The importance of Wnt signaling is not restricted to the early stages of mammogenesis (Incassati *et al*. 2010); rather, it continues to play a role in the further development of the rudiments. Protein produced from the *WNT10A* gene plays a role in the development of many parts of the body. It appears to be essential for forming tissues that arise from an embryonic cell layer called the ectoderm (https://medlineplus.gov/genetics/gene/wnt10a/). Though the loss of Wnt 5A does not alter embryonic mammary gland induction (Chu *et al.* 2004), it accelerates and enhances ductal growth during hormonal development by increasing lateral branching and TEB size (Roarty and Serra, 2007).

The candidate gene approach is a valuable tool to determine the association of a genetic variant with a disorder and to identify the genes of modest effect (Kwon and Goate, 2000). We hypothesized that regarding the highlighted molecular function of *WNT10A* for the formation and maintenance of the mammary bud, early development of the embryo and highly associated with numerous cancers and act as oncogenes or tumor suppressor genes, identification of polymorphism in this gene make the potential for possible association study. When comparing our results to those of older studies, it must be pointed out that the selection of candidate genes is followed by the identification of polymorphisms within the gene and the analysis of the association of the genotype at the candidate gene loci with the phenotype (Stratil and Geldermann, 2004). Our results reveal the same allelic variation (A/G exon 4) between two experimental groups. Very little was found in the literature on the Wnt10A gene in sheep and automatic discussion and comparison of our outputs affect this deficiency of knowledge. Prior studies have reported the importance of other discovered candidate genes in teat number variation and SNT like, Relaxin1(RLN1) (Un, 2002), estrogen receptor, insulin-like growth factor 2 (Oltmanns, 2003), follicle-stimulating hormone beta (Oltmanns, 2003), thyroid hormone beta, parathyroid like hormone gene, leptin receptor, prolactin, fibroblast growth factor (FGF2), epidermal growth factor (EGF), leucinef- rich G protein-coupled receptor, growth hormone receptor (Un, 2002), Insulin-like growth factor (Un, 2002), Growth hormone-releasing hor

mone receptor (Un, 2002) are summary of a listed candidate gene for teat number and the presence of supernumerary teat in livestock (Wysolmerski *et al.*1998; Foley *et al.* 2001).

Statistically, we used the database for annotation, visualization and integrated discovery (DAVID), kyoto encyclopedia of genes and genomes (KEGG), stimulator of interferon genes (STRING) and Gene Function Prediction using a multiple Association Network Integration Algorithm (gene Mania) computational free tools and online bioinformatics resource and demonstrated this critical role path in mammary gland development during embryonic ectodermal organs differentiation process and formation of teats. Stimulation of the pathway leads to a series of biological PI3K, STAT, transforming growth factor β, epidermal gro-cellular pathways such as fibroblast growth factor, MAPK, wth factor, Parathyroid hormone-related protein, ectodysplasin pathways (Figures 7-9).

In summary, the present result highlights no significant association between identified gene-based variation in the investigated candidate genes and supernumerary teat in Ghezel and Romanov sheep breeds.

Limitations of the present studies naturally include a low sample size of animals that carry supernumerary four functional teats and only one candidate gene for biomarker inquiries, so future investigations are necessary to re-perform such motivation using more sample size, more prominent candidate gene and sophisticated technology such as next-generation sequencing and genome-comprehensive association projects.

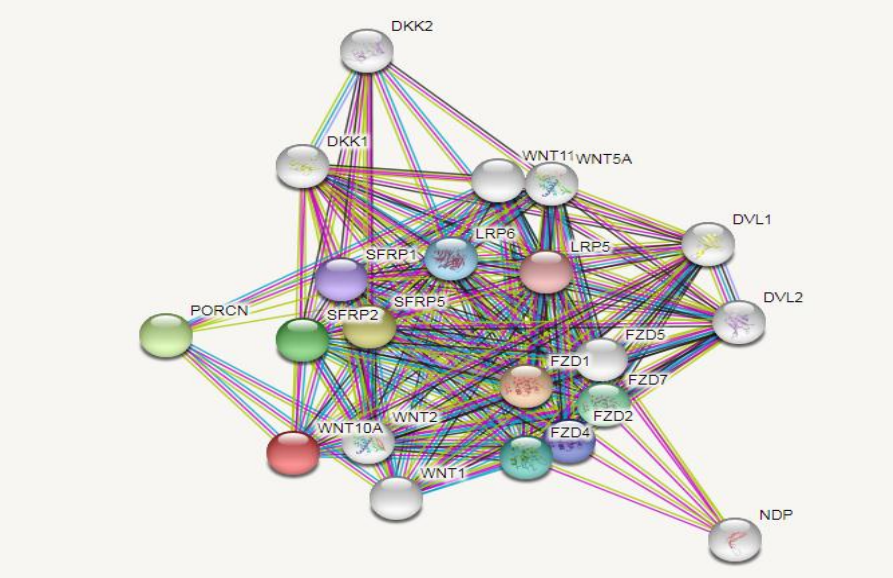

Figure 7 *WNT* gene networking using cytoscape software

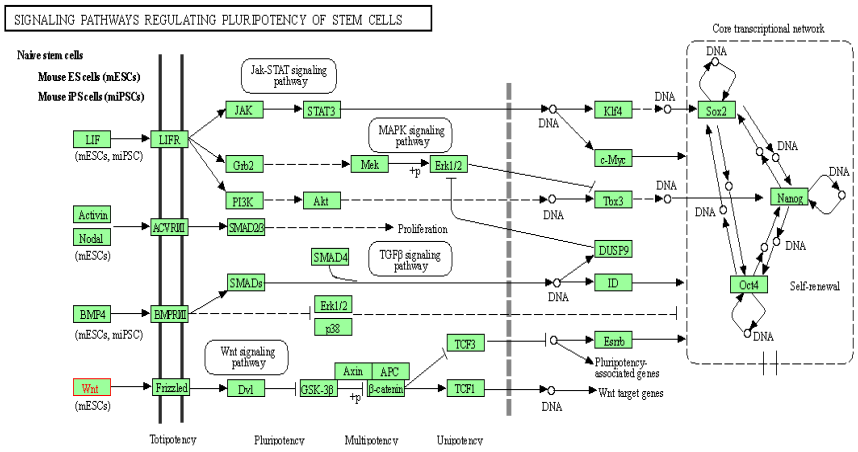

Figure 8 *WNT* gene ontology and networking using KEGG software

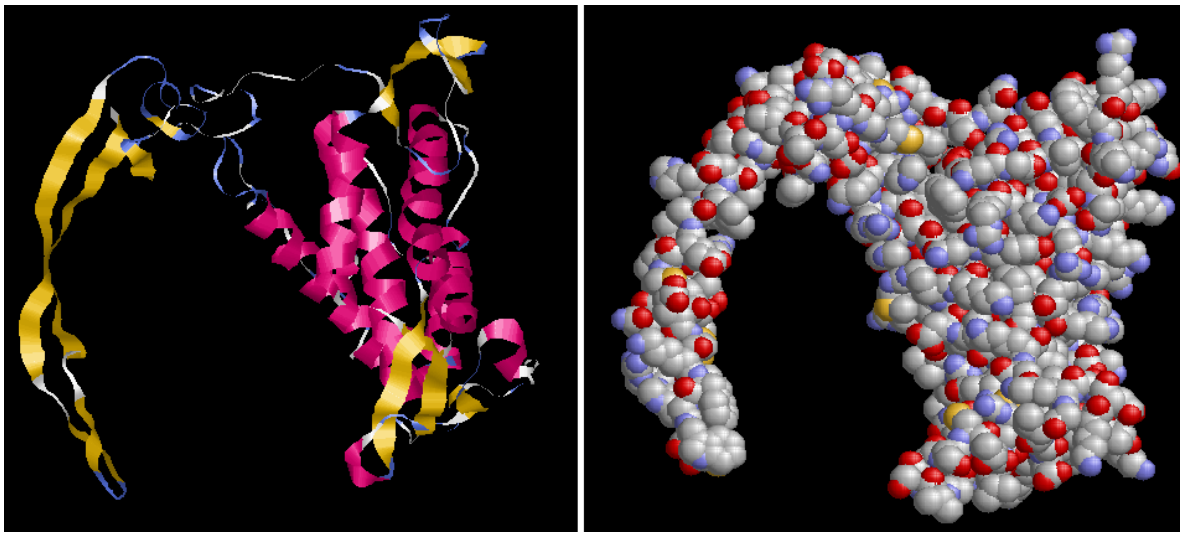

Figure 9 WNT protein using 3D modeling perspective

CONCLUSION

On this basis, our study addressed the frequency of four healthy teats differed among investigated breeds and ranged from 2 to 10% in ROM and GHE sheep, respectively. For the *WNT10A* gene, no evidence of allelic variation (A/G exon 4) between the case and control group in GHE and ROM was detected. Overall, our study provides a preliminary attempt to linked candidate gene polymorphism to a SNT in GHE, which may confirm the critical role of this gene in the mammary epithelium differentiation during embryonic programming for future lactation.

ACKNOWLEDGEMENT

The authors would like to acknowledge Dr. András Gáspárdy from the Department for Genetics and Animal Breeding, Szent István University for sharing his Ph D. student soft copy of the insightful dissertation and valuable profound experiences about the prevalence of supernumerary teat in livestock.

REFERENCES

Abedini A., Zamberlam G., Boerboom D. and Price C.A. (2015). Non-canonical WNT5A is a potential regulator of granulosa cell function in cattle. *Mol. Cell. Endocrinol.* **403,** 39-45.

Adebayo J. and Chineke C. (2011). Evaluation of West African dwarf goat for some qualitative traits in Southwestern Nigeria. *African J. Agric. Res.* **6,** 6204-6207.

Amao O., Osinowo O., Lakpini C., Dipeolu M., Abiola S. and Onwuka C. (2003). Types and frequency of udder shapes and abnormalities in West African Dwarf and Red Sokoto goats. *Nigerian J. Anim. Prod.* **30,** 253-258.

Bemji M. and Popoola S. (2011). A note on the incidence of udder abnormalities in West African Dwarf goat in South Western Nigeria. *Livest. Res. Rural Dev.* **23,** 3-11.

Brka M., Reinsch N. and Kalm E. (2002). Frequency and heritability of supernumerary teats in German Simmental and German Brown Swiss cows. *J. Dairy Sci.* **85,** 1881-1886.

Chu E.Y., Hens J., Andl T., Kairo A., Yamaguchi T.P., Brisken C., Glick A., Wysolmerski J.J. and Millar S.E. (2004). Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. *Development*. **131,** 4819-4829.

Foley J., Dann P., Hong J., Cosgrove J., Dreyer B., Rimm D., Dunbar M., Philbrick W. and Wysolmerski J. (2001). Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. *Development*. **128,** 513-525.

Incassati A., Chandramouli A., Eelkema R. and Cowin P. (2010). Key signaling nodes in mammary gland development and cancer: β-catenin. *Breast Cancer Res.* **12,** 213-221.

Kenny S. (2014). Investigation of prevalence of supernumerary teat in livestock. MS Thesis. Oregon State Univ., United States.

Kwon J.M. and Goate A.M. (2000). The candidate gene approach. *Alcohol Res. Health*. **24(3),** 164-172.

Lundeheim N., Chalkias H. and Rydhmer L. (2013). Genetic analysis of teat number and litter traits in pigs. *Acta Agric. Scandinavica A-Anim. Sci.* **63,** 121-125.

Martin P., Palhière I., Tosser-Klopp G. and Rupp R. (2017). Corrigendum to "heritability and genome-wide association mapping for supernumerary teats in French Alpine and Saanen dairy goats. *J. Dairy Sci*. **99,** 8891-8900.

Oltmanns I. (2003). QTL-analyse an den Chromosomen 2, 3, 5, 7, 9, 11, 13, 15 und 17 in einer porcinen ressourcepopulation. MS Thesis. University of Bonn, Bonn, Germany.

Oseni S., Sonaiya B., Omitogun G., Ajayi A. and Ishola M. (2006). West African Dwarf Goat Production under Village Conditions: 1. Characterization and the Establishment of Breed Standards. Pp. 1-5 in Proc. Conf. Int. Agric. Res. Dev., Bonn, Germany.

Ozoje M. (2002). Incidence and relative effects of qualitative traits in West African Dwarf goat. *Small Rumin. Res*. **43,** 97-100.

Pausch H., Jung S., Edel C., Emmerling R., Krogmeier D., Götz K.U. and Fries R. (2012). Genome wide association study uncovers four QTL predisposing to supernumerary teats in cattle. *Anim. Genet*. **43,** 689-695.

Roarty K. and Serra R. (2007). Wnt5a is required for proper mammary gland development and TGF-β-mediated inhibition of ductal growth. *Development*. **134,** 3929-3939.

Stratil A. and Geldermann H. (2004). Analysis of porcine candidate genes from selected QTL regions affecting production traits. *Anim. Sci. Pap. Rep.* **1,** 23-27.

Ün C. (2002). QTL-Analyse an Chromosomen 1, 4, 6, 8, 10, 12, 14, 16 und 18 in einer porcinen resourcenfamilie. MS Thesis. University of Bonn, Bonn, Germany.

Vainikainen V. (1945). On the heritability of supernumerary nipples in Finnish home-bred sheep. *Agric. Food Sci.* **17,** 11-18.

Wysolmerski, J.J., Philbrick, W.M., Dunbar M.E., Lanske, B., Kronenberg H. and Broadus A.E. (1998). Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development*. **125,** 1285-1294.