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Allelic polymorphism of exon 2 in *BMP15* gene in F1 crossbred sheep from crossing Romanov rams with Kermani ewes

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Abstract This study was carried out to investigate the polymorphisms of bone morphogenetic protein 15 (*BMP15*) exon 2 in purebred Kermani and crossbred Romanov × Kermani sheep and functional analysis of the underlying mutations. A number of 50 purebred Kermani and 115 F1 Romanov (ram) × Kermani (ewe) crossbred sheep were sampled and a 153 bp fragment from exon 2 of the ovine *BMP15* gene was successfully amplified from the genomic DNA of each animal using designed primers. The polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) technique was used to investigate the polymorphism of *BMP15* gene (exon 2). Twenty samples of different SSCP patterns were randomly selected for DNA sequencing and detecting the *BMP15* mutations and subsequent functional analyses. The polymorphic fragments amplified by designed primers were sequenced. There were eight SSCP patterns (*AA*, *AB*, *BB*, *AC*, *AD*, *AE*, *AF* and *AG*) with frequencies of 0.24, 0.17, 0.24, 0.08, 0.05, 0.10, 0.03 and 0.09, respectively. The frequency of *A* allele was obviously higher than those of other alleles. The sequencing results revealed two single nucleotide mutations; the first mutation at position 32bp which did not cause any change in the amino acid sequence but the second mutation led to a change in 40th amino acid (a Lysine amino acid replaced by an Asparagine amino acid). The result of this experiment indicates that the genetic diversity level of *BMP15* exon 2 gene was high in the Romanov × Kermani crossbreds indicating that *BMP15* exon 2 can played a vital function in the development of ovary and follicles, especially in the improvement of fertility trait and could be used as a potential advantageous molecular marker for reproduction traits in this genetic group. Our finding provides exciting new opportunities for understanding the role of the *BMP15* on ovarian follicular growth and development in crossbreed ewes in breeding programs. However, further investigation using a large population of Romanov × Kermani crossbred sheep is required to confirm the link between the identified mutation and the observed increased prolificacy in this population.

Keywords: *BMP15*, PCR-SSCP, polymorphism, Romanov × Kermani sheep

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Introduction

Reproduction and fertility traits have a major impact on efficiency and profitability in lamb meat production (Rosati et al., 2002). Ovulation rate and litter size are important fertility traits in sheep and are of high economic value (Notter, 2008). Traits associated with fertility usually have low heritability, and breeding improvements made on phenotypic selection based on observable data are often limited (Safari et al., 2005). On the other hand, ovulation rate and litter size are also only expressed in one sex and are recordable only relatively late in the animal's life. This further hinders the breeding progress and limits the inclusion of the traits into selection schemes since selection of breeding candidates is complicated. Hence, it is important to study genes associated with fertility traits so that breeding can include genotypic information from animals (Snowder and Fogarty, 2008). This will increase the genetic improvements in reproduction traits since it will be easier to collect data and information on such traits. Focusing on development of fertility traits, using molecular information, will have a long term effect on profitability in the sheep production. Knowledge of the genes associated with reproduction, especially those related to ovulation and litter size, is important. Identifying genes with major effect is also a prerequisite which offers the opportunity to improve the reproductive efficiency (Esmailizadeh, 2010; Esmailizadeh, 2014; Nosrati, et al., 2019), and product quality and product diversity in livestock industry (Purvis and Franklin, 2005). Three fecundity major genes belonging to the TGFβ superfamily, have been identified in sheep; namely, *BMPR-1B* (bone morphogenetic protein receptor 1B), *BMP15* (bone morphogenetic protein 15) and *GDF9* (growth differentiation factor 9) which are located on chromosomes 6, X and 5 in sheep, respectively (McNatty et al., 2001; Hanrahan et al., 2004).

So far, eight genetic mutations with major effects on ovulation rate and litter size have been identified in *BMP15* gene; including, $FecX^{I}$ (Inverdale), $FecX^{B}$ (Belclare), $FecX^{G}$ (Cambridge), $FecX^{H}$ (Hanna), $FecX^{L}$ (Lacaune), $FecX^{R}$ (Rasa Aragonesa) and $FecX^{Gr}$, $FecX^{O}$ (Grivette and Olkuska sheep). However, these mutations are not applicable to all breeds of sheep (Davis, 2005). Since *BMP15* plays an important role in prolificacy and fertility, it is important to know the sequences of this gene in different sheep breeds. A good knowledge of the sequences of this gene will help in identifying the variants responsible for the observed variations in prolificacy and fertility.

Kermani sheep is an indigenous fat-tailed sheep breed in southeast of Iran. Similar to most of the local Iranian sheep, almost all Kermani ewes produce singleton lambs (Mokhtari et al., 2010), mainly because of the selection system which is based on extensive grazing and pastoralism where only single lambs could mostly survive. The Romanov sheep breed is famous for its high prolificacy; including multiple births, capability to lambing twice annually and out-of-season estrus. Recently, the Romanov rams have been imported to Iran for crossing with the native breeds including Kermani sheep to increase the flock fecundity. However, the molecular mechanisms underlying high prolificacy in Romanov breed remain mainly unknown and there is no available genetic solution to select for prolificacy in the Romanov×Kermani sheep crossbred ewes while preserving the native breed adaptation to the local environmental conditions. Therefore, the objective of this study was to investigate the molecular genetic variation of *BMP15* gene (exon 2) of pure Kermani sheep and their crosses with Romanov rams to provide relevant molecular information for marker-assisted selection in the crossbred Romanov×Kermani sheep. Therefore, we investigated the presence of single nucleotide polymorphisms (SNPs) within exon 2 of *BMP15* gene using PCR-SSCP and DNA sequencing methods and used bioinformatics tools to further study the functionality of the identified SNPs.

Materials and methods

Animals and sampling

Jugular blood samples (5 mL) were collected from 50 purebred Kermani and 115 F1 Romanov (ram) × Kermani (ewe) crossbred sheep in EDTA coated tubes, immediately placed on ice and transported to the Molecular Genetics Laboratory, Department of Animal Science, Shahid Bahonar University of Kerman, Iran, and were stored at -20 °C until the DNA extraction. Genomic DNA was extracted from leucocytes, using salting out method. The DNA samples were dissolved in Elution buffer (pH= 8.0) and stored at -20 °C for further analysis.

Primer synthesis and PCR reaction

Recovery and purity of each DNA sample was estimated by UV spectrophotometry. Separation and purification of DNA fragments were done by electrophoresis through 1% agarose gel. Electrophoresis was carried out at 7 to 8 volt/cm of the gel, and the migration was monitored using UV transilluminator. A pair of primers was designed to amplify a 153-bp fragment in exon 2 of the

BMP15 gene. The primers (Table 1) were designed based on forward primer of Hanrahan et al. (2004) using the Primer 3 online software (http://www.simgene.com /Primer3).

Based on the methods described by Hanrahan et al. (2004) , the PCR-SSCP was used to detect the *BMP15* mutations. In this method, a point mutation is deliberately introduced into one of the primers. DNA samples were adjusted to a concentration of 50 ng/µL and exactly 2 µL of the DNA samples were used as template for polymerase chain reaction (PCR). Total volume of 26 µL of each PCR reaction contained 2 µL of genomic DNA, 2 mM of $MgCl_2$ (0.8 µL of a solution containing 2.5 mM $MgCl_2$ per µL), 1 µL (10 µM) of each of forward and reverse primers, 0.5 µL (5 µM) of dNTPs, 1 unit (0.2 µL) of Taq DNA polymerase, 3 µL of 10 × PCR buffer and 17.5 µL deionized water. The amplification of the 153-bp fragment in exon 2 of the *BMP15* gene was performed with an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s; annealing at 63°C for 30 s, extension at 72°C for 15s, then holding at 72°C for 10 min. Aliquots of 5 µL PCR products were mixed with 5µL denaturing solution (98% formamide, 0.025% xylene-cyanole and 0.025% bromophenol blue, 10 mM EDTA), incubated at 98°C for 10 min and then chilled on ice. Denatured DNA was loaded on 10% PAGE gel (80 mm×73 mm×0.75 mm) in 1×TBE buffer and constant voltage 200V for 2.5 h. The gel was stained with 0.1% silver nitrate solution.

The genotype and allele frequencies in two genetic groups were calculated. The Hardy-Weinberg equilibrium (HWE) for the loci in the population was tested using the POPGENE software Ver. 3.2 (Yeh et al., 1997). In this study, we calculated the population genetic diversity indexes including; expected gene heterozygosity (He); $He = 1 - \sum_{i=1}^{n} p_i^2$, effective number of alleles (Ne); $Ne=1/\sum_{i=1}^{n} p_i^2$, Nei's diversity index (H_S) which measures the average gene diversity per locus (Nei, 1973) and is determined by the formula; $H_S=\frac{1}{k}\sum_{s=1}^{k}[1-q_s^2-(1-q_s)^2]$, where k is the total number of loci (differentiating factors), and q_s is the frequency of one of the two alleles at the s*th* diallelic locus.

The Shannon diversity index (H_I) was another criterion used to characterize the population genetic diversity, according to the formula; $H_I=-\sum_{i=1}^{k} p_i log(p_i)$, in which *pi* denotes the proportion in group k.

Sequence analysis

Twenty samples from different SSCP patterns were randomly selected for DNA sequencing. The primers used for sequencing were the same as those for the PCR reaction. The PCR products were sequenced (Gene Fanavaran Co., Iran). The observed sequences of the studied 153-bp fragment of exon 2 in the *BMP15* gene were compared with a sequence reported for North African sheep (GenBank No. GU117618). The nucleotide sequences were translated to the codified AA sequences using the tools developed by the Swiss-Prot group (https://www.expasy.org/resources/translate). Secondary structures of the proteins were predicted using protein section of DNASTAR software (DNASTAR Inc., Madison, WI. USA). Moreover, 3D structures of proteins were predicted using Molegro Molecular Viewer 2.2 (https://molegro-molecular-viewer.software.informer.com/2.2/)

Results and discussion

The genomic DNA was successfully isolated from blood samples. The gel electrophoresis pattern of different samples (Figure 1) indicated the good quality of the ex-

Table 1. Primer sequence and annealing temperature of the assays

Gene	No.	Primer sequence (5'→3')	Annealing temperature (°C)
BMP15	F	5'-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC	63
	R	5'-GATGCAATACTGCCTGCTTG	

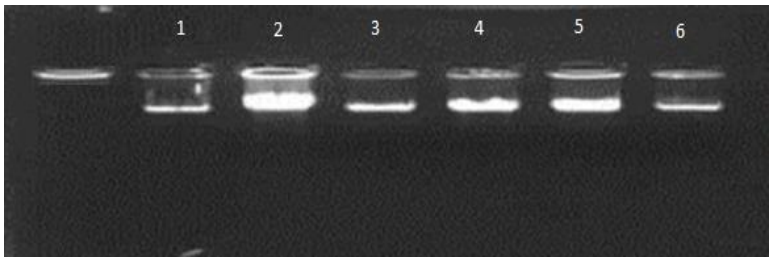

Figure 1. Genomic DNA isolated from Romanov × Kermani sheep (Lanes 1-6: Genomic DNA, first lane is the negative control).

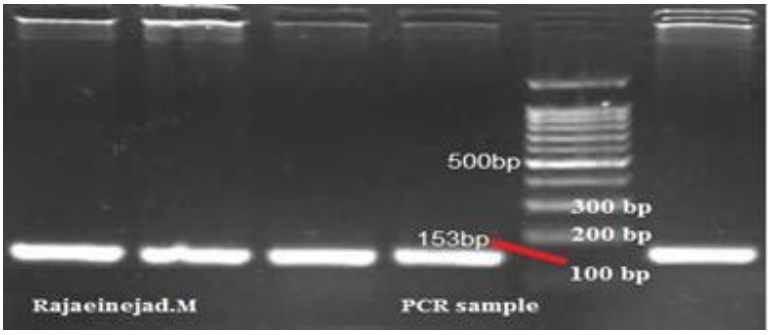

Figure 2. PCR products of *BMP15* gene, in Romanov × Kermani sheep breed analyzed by electrophoresis in 1% agarose gel.

tracted DNA.

In the present study, PCR-SSCP was used to investigate the polymorphism of BMP-15 gene (exon 2). Following adding the primers, the agarose gel electrophoresis was used to assess the quality of extracted DNA. The PCR products were separated by 1% agarose gel and the sharp and clear bands without smears were observed (Figure 2). The results indicated that, the 153 bp fragment from exon 2 of the ovine *BMP15* gene was successfully amplified from the DNA of each sample (165 samples) in pure Kermani and Romanov × Kermani sheep.

The single strand conformation polymorphism technique, which is an effective procedure for detecting the amplified sequence diversity (Min et al., 2005) was used to detect mutation variations in the amplified fragments. After optimization of the parameters which affect the detection of SSCP, the PCR products from the 165 animals were analyzed as described before. The results revealed seven alleles (named A and B, C, D, E, F and G), identified and designated on the basis of electrophoretic mobility in the gel. The identified conformational patterns (genotypes) included: AA, AB, BB, AC, AD, AE, AF and AG (Figure 3).

While two point mutations in exon 2 of *BMP15* were genotyped by PCR-SSCP based methodology, as per expectations for exon 2 of *BMP15* in Romanov × Kermani crossbred, no polymorphisms were detected at the exon 2 of *BMP15* gene in Kermani native sheep breed, and the individuals were monomorphic for exon 2 of *BMP15* gene (Figure 4). All the PCR products were well resolved and sized by agarose gel electrophoresis, allowing easy identification of different genotypes. Heterozygotes and homozygotes were unambiguously assigned from the gel profile. The size of DNA fragments, amplified (153bp fragment of exon 2) with the designed

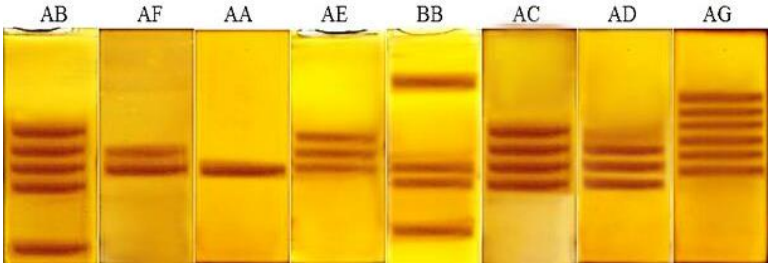

Figure 3. The SSCP analysis of PCR products and genotypic patterns of *BMP15* gene (exon 2) for Romanov × Kermani crossbred sheep.

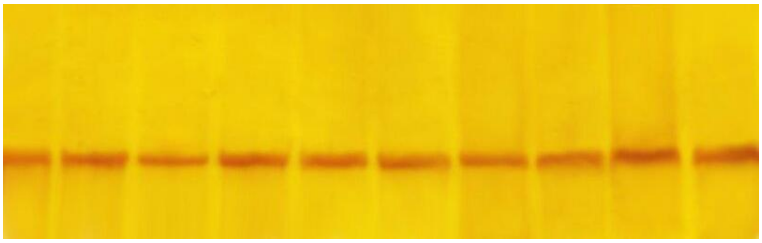

Figure 4. An agarose gel electrophoretogram for *BMP15* (exon 2) loci product showing monomorphic genotypes in pure Kermani sheep. All individuals showed monomorphic allele with 153 bp in length.

primers for 500 bp control fragment, was suitable for separation on 1% agarose gels (Figures 3 and 4).

Given that, the studied locus was found to be polymorphic in crossbred sheep, the calculated genotype frequencies for AA, AB, BB, AC, AD, AE, AF and AG in Romanov × Kermani crossbreds were 0.24, 0.17, 0.24, 0.08, 0.05, 0.01, 0.09 and 0.03, respectively. The AA and BB genotypes were the most frequent genotypes. Also, allelic frequencies for the A, B, C, D, E, F and G were 0.5, 0.325, 0.0417, 0.0250, 0.005, 0.0425 and 0.0125, respectively, and the A allele had the highest frequency. The designed primers produced well defined discrete banding pattern which revealed eight genotypes based upon the presence or absence of the alleles. The absence of an amplification product with these primers in an individual was considered as missing data. Observed and average heterozygosity values for the locus were quite high, 0.53 and 0.64 respectively. The observed number of alleles per locus, Shannon (H_I) and Nei's diversity indices (H_S) that are good indicators of the genetic diversity (Allen et al., 2009), were 7, 1.28 and 0.64, respectively, for the studied population (Table 2). Given the estimated parameters and rather the high degree of het erozygosity, it can be concluded that a relatively high polymorphism exists the studied population. It is generally expected that the crossbred ewes are more prolific.

Table 2. Summary of the genetic diversity criteria within the exon 2 of *BMP15* gene in Romanov × Kermani crossbred sheep

Number of actual alleles	7
Expected heterozygosity	0.640
Observed heterozygosity	0.530
Average heterozygosity	0.640
Expected homozygosity	0.35
Observed homozygosity	0.64
Shannon diversity Index(H_I)	1.28
Nei's diversity index (H_S)	0.640
Number of effective alleles	2.78

The results obtained from the SSCP analysis were confirmed by sequencing of twenty randomly selected DNA samples. The sequencing results showed two point mutations in 32bp and 95bp positions of the exon. Alignment of the sequences from Romanov × Kerman crosses with those obtained from Gen-Bank (GU117618) by Vector NTI software (https://vector-nti.software.informer.com/11.0/) revealed a guanine (G) by adenine (A) substitution at the 95bp position leading to an exchange in the asparagine (Asn) with lysine (Lys) amino-acid (Figures 5 and 6). Our study showed presence of one synonymous substitution (A to C) which do not alter amino acid sequences at 32bp position in the *BMP15* exon 2.

After nucleotide alignment, the DNA sequences were translated into protein sequences. The alignment of the Romanov × Kermani sheep protein sequence with the protein sequence of North African sheep revealed that nucleotide changes at position 32 did not change the amino acid sequence. In fact, the single nucleotide changes in the situation created by this kind of synonymous codon do not change the peptide sequence. However, changes in amino acid sequence occurred at the nucleotide position 95 (guanine to adenine nucleotide transposition). In the alignment process, the lysine amino acid (K) at the position 40 was replaced with asparagine amino acid (N) (Figure 6). By translating the sequence, it was found that the amino acid at position 40 is displaced; the displacement of amino acid lysine to asparagine (a polar charged amino acid (K) was replaced

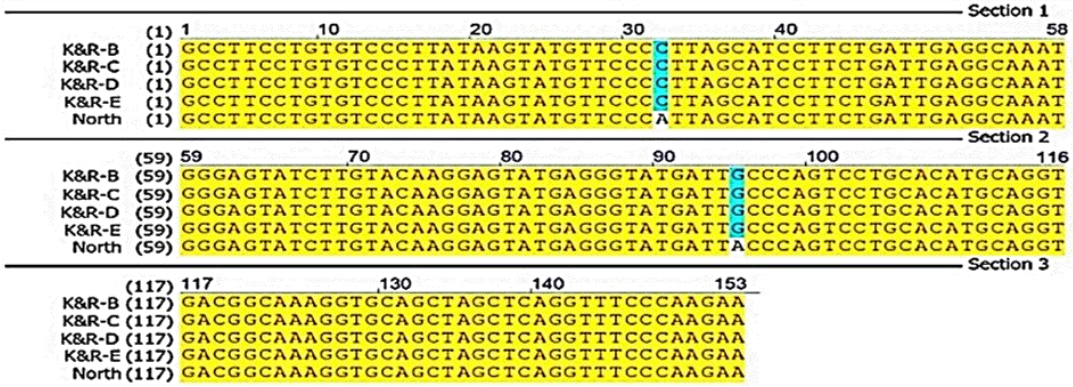

Figure 5. Alignment of Romanov × Kermani (K&R) crossbred sequences with sequences taken from Genbank (GU117618). The point mutations of "A to C" and "G to A" were found at positions 32bp and 95bp of the amplified 153-bp fragment of *BMP15* gene exon 2. "K&R" and "North" denote Romanov × Kermani and North African sheep, respectively.

	1	1 10 20 30 40 50
K&R-B	1	LGKPELAAPLPSPACAGLGNHTLILLVQDTPICLNQKDAKGNILIRDTGR
K&R-C	1	LGKPELAAPLPSPACAGLGNHTLILLVQDTPICLNQKDAKGNILIRDTGR
K&R-D	1	LGKPELAAPLPSPACAGLGNHTLILLVQDTPICLNQKDAKGNILIRDTGR
K&R-E	1	LGKPELAAPLPSPACAGLGNHTLILLVQDTPICLNQKDAKGNILIRDTGR
North	1	LGKPELAAPLPSPACAGLGNHTLILLVQDTPICLNQKDANGNILIRDTGR

Figure 6. The codified protein sequence and deduced amino acid exchange of *BMP15* exon 2 protein in Romanov × Kermani sheep. Numbers at the start of each line indicate amino acid positions in the full-length unprocessed protein. K: Lysine amino acid; N: Asparagine amino acid; "K&R" and "North" denote Romanov × Kermani and North African sheep breeds, respectively.

by a polar amino acid without load (N)).This replacement affects the dimer formation and causes the changes in electrical potential at the displacement surface (Hanrahan et al., 2004).

The results indicated that the changes in the allele A in comparison to the allele G, cause a different predicted 3D protein structure. The type of the mutation observed in this study was a point missense mutation in which a single nucleotide change results in a codon that codes for a different amino acid. If the replaced amino acid has different properties, the possibility of changing protein's performance is high. For example, sickle-cell disease is caused by a single point mutation (a missense mutation) in the beta-hemoglobin gene that converts a GAG codon into GUG, which encodes the amino acid valine rather than glutamic acid (Clancy, 2008; Rees et al., 2010). In this study, the lysine amino acid (a polar positively charged amino acid) substitutes with an asparagine (a polar and uncharged amino acid) amino acid. Lysine frequently plays an important role in protein structure. Since its side chain contains a positively charged group on one end and a long hydrophobic carbon tail close to the backbone, lysine is considered somewhat amphipathic (Betts and Russell, 2003). It thus mostly prefers to substitute for the other positively charged amino acid, arginine, though in some circumstances it will also tolerate a change to other polar amino acids. Modeling of the mutation in Romanov × Kermani sheep, in terms of loaded charge, was very similar to the $FecX^{I}$ (in Inverdale sheep) and $FecX^{O}$ (Polish Olkuska sheep). In $FecX^{I}$, a substitution of C to T nucleotide, changes the positive-charged hydrophobic valine to the negative-charged aspartic acid and in $FecX^{O}$, an uncharged polar asparagine is replaced by an uncharged polar histidine (Davis, 2005; Montgomery et al., 2001). Prediction of the three-dimensional structure of the protein sequence indicated that the substituted lysine amino acid tended to assemble on the outer part of the protein structure, where it can interact with water (Figure 7).

The replacement of a charged amino acid with an uncharged one, may affect the dimer formation of proteins leading to changes in the electrical potential. Same as most members of the transforming growth factor-β (TGF-β) superfamily, the BMP15 is thought to be biologically active as dimers. However, it is not known whether the physiologically active dimers are homodimers (GDF9-GDF9 and BMP15-BMP15), or heterodimers (GDF9-BMP15), whether all three dimer forms play a role, or whether different combinations are predominant in different species. It is still unclear whether GDF9 and BMP15 act separately or together in sheep, but previous observations (Hanrahan et al., 2004; McNatty et al., 2004; Polley et al., 2010) showed that the effects of a GDF9 mutation and a BMP15 mutation together in one animal appear to be additive, implying that the GDF9 and BMP15 proteins are likely to be working independently.

The study of fecundity genes is of great importance in the sheep industry (Nicol et al., 2009). The identification and use of major fecundity genes in sheep production will enable an increase in reproductive traits and thus an increase in genetic improvement. Additionally,

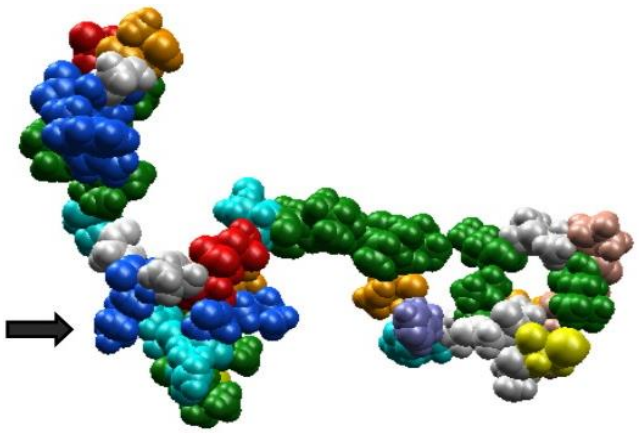

Figure 7. The changes of 3D structures of protein predicted for the alleles G and A of the protein sequence of BMP15; the arrow shows lysine position in the referred peptide for BMP15 protein.

an increase in ovulation rate and litter size will result in higher productivity of the ewe, leading to more efficient lamb production. The proteins of the transforming growth factor-β (TGF-β) superfamily have important roles in the intraovarian regulation of follicular development (Juengel and McNatty, 2005). Different mutations in the genes from the TGF-β superfamily have been shown to affect ovulation rate and litter size in sheep. These genes include *BMPR-1B*, *BMP15*, and *GDF9* (Galloway et al., 2000; Galloway et al., 2002; Davis, 2005). In our study, we identified one novel heterozygous missense and a synonymous mutation in Romanov × Kermani crossbreds that could be implicated in the ovarian function. Also, the absence of these variants in the purebred Kermani sheep suggests a potential effect of the *BMP15* (exon 2) polymorphisms in fertility traits in the crossbreds. Synonymous mutations do not change the sequence or structure of the protein, once was thought to be functionally neutral, but evidence now indicates it is shaped by evolutionary selection and affects other aspects of protein biogenesis beyond specifying the amino acid sequence of the protein (Bali and Bebok, 2015). Analyzing the consequences of synonymous codon changes in different organisms has revealed that they impact nucleic acid stability, and protein levels, structure and function without altering the amino acid sequence (Chaney and Clark, 2015). Furthermore, gene expression is correlated with synonymous codon usage bias. Thus, the codon choice may affect the *BMP15* gene expression or the coordinated expression of functionally related genes, further affecting the follicle development, ovulation and litter size. However, much remains unknown about the molecular mechanisms connecting the synonymous codon usage to efficient protein biogenesis and proper cell physiology.

In conclusion, the genetic diversity level within exon 2 in the *BMP15* gene was high in the Romanov × Kermani crossbreds, indicating that BMP15 protein can play a vital function in the development of the ovary and follicles, especially in the improvement of fertility trait, and could be used as a potential advantageous molecular marker for reproductive traits in this genetic group. Our finding provides exciting new opportunities for understanding the role of the oocyte-secreted factor *BMP-15* on ovarian follicular growth and development in crossbreed ewes in breeding programs. However, further investigation using a large population of Romanov × Kermani crossbred sheep is required to confirm the link between the identified mutation and the observed increase in prolificacy in this population.

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