Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v45i1.57287

Association of Palm gene mutations with structure and function of paralemmin proteins in Lori-Bakhtiari and Zel sheep Breeds

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ABSTRACT. The aim of current study was to survey genetic variability of PALM gene's exon 3 and 4 by PCR-SSCP and DNA sequencing in Zel and Lori Bakhtiari sheep breeds. The SIFT (Sorting Intolerant from Tolerant) and PHyre2 program were used to predict the possible impact of amino acid substitutions on performance and structure of the paralemmin protein. A total of 140 animal's from 2 Iranian sheep breeds with different fat metabolisms, Lori-Bakhtiari and Zel sheep breeds were considered. The results showed that there are two polymorphic sites including a nonsynonymous substitution and an insertion mutation (49bp). Non-synonymous mutation deduced Thr20Ala amino acid exchange and ensuing two different structures for paralemmin protein that could be potentially affect protein structure and function during the interaction with glutamate in the cytosolic surface of plasma membrane. PALM gene, according to evolutionary path, is classified into two separate categories. In first covey, *Gallus gallus* and in second one, other species in several branches, so that the sequence of cow and sheep is placed in a sub-branch which forms a clade beside goat. Comparison of illustrated coding region sequences, PALM gene among different species, is of orthologous which are derived from a common ancestor.

Keywords: Paralemmin; polymorphism; protein structure; fat and thin tail breed.

Received on January 3, 2021.
Accepted on September 20, 2021.

Introduction

Sheep (*Ovis aries*) is the first grazing animal known to have been domesticated (Hu, Petrasch-Parwez, Laue, & Kilimann, 2005). Multiple mitochondrial lineages suggested sheep domestication occurred several times, as in other livestock species such as cattle, goat and pig (Moradi, Nejatip-Javaremi, Moradi-Shahrbabak, Dodds, & Mc Ewan, 2012). Fat tail breeds are an important class of sheep breeds that are first documented as being present 5000 years ago. Approximately 25% of the world sheep population comprises fat tail breeds that are grazed in a wide region of countries especially in Asia and northern parts of Africa. The fat tail is considered as an adaptive response of animal to a hazardous environment and is a valuable energy resource for the animal during migration and winter (Khaldari et al., 2020). Sheep production constitutes the most important component of the Iranian livestock industry whit a total of approximately 50 million head. Twenty seven breeds and ecotypes have been documented in Iran. In livestock industry, the selection of animals based on their growth rate and body composition is favored for production of higher quality meat. Molecular techniques allow detecting variation or polymorphisms exists among individuals in the population for specific regions of the DNA. These polymorphisms can be used to build up genetic maps and to evaluate differences between markers in the expression of particular traits in a family that might indicate a direct effect of these differences in terms of genetic determination on the trait (Albert et al., 2007).

The polymorphism in marker loci is the most useful tool for detection of economically important characteristics. As pointed out by Moradi et al. (2012) on selection sweep signatures using F_{ST} in thin and fat tail breeds (sampled from the Ovine HapMap project), confirmed three of these regions located on chromosomes 5, 7 and X. Using a median run of homozygosity plot in these regions, they identified that homozygosity has been increased on chromosome 5 and X in favor of fat tail breeds and on chromosome 7 in

favor of thin tail breeds. Those associated with an increase of homozygosity in the fat tail breed would be consistent whit selection for mutations affecting fat tail size several thousand years after domestication (Moradi et al., 2012).

Fat deposition requires more energy than the deposition of lean tissue, animal fat has lost much of its market demand and monetary value and sheep producers have easy access to other forms of auxiliary feeding (Moradi et al., 2012). To data, several investigations into the inheritance of fat tails have been undertaken (Edwin, Wong, Goodship, & Kavanagh, 2013), nevertheless the genes affecting fat deposition in fat tail breeds are still unknown (Moradi et al., 2012). We found few genes in the region of interest and no particular candidate genes related to fat deposition were identified. PALM gene is an candidate gene for this observation located on chromosome 5 (chr5 :47146931-47175489) of the sheep. The ovine PALM gene is located on chromosome 7, having nine exons and 8 introns and located on chromosome 5 sheep. Paralemmin is a prenyl-palmitoy anchored membrane protein that can drive membrane and process formation in neuron. Paralemmin are a protein family whit four previously characterized isoforms in animal: PALM1, PALM2, PALM3 and PALMD (palmdelphin). The first three are anchored to the plasma membrane through prenylation and di-palmitoylation of a C-terminal cysteine cluster (CaaX motif), whereas palmdelphin is predominantly expressed as a splice variant that lacks the CaaX motif, thereby becoming a cytosolic protein. In the lens, palm is already expressed at 9.5 dpc in the lens placode, and this expression is maintained in the lens vesicle throughout the formation of the adult lens. The expression pattern of palm in the eye is consistent with it being a Pax6 responsive gene (Castellini et al., 2005; Hindorff et al., 2009).

Paralemmin (PALM), encodes a protein present at the plasma membrane in axons, dendrites and perikarya of differentiating neuronal cell lines, and at high levels in the processes of the cerebellar molecular layer (Hu, Copeland, Gilbert, Jenkins, & Kilimann, 2001). Paralemmin associated with lipid rafts and have been proposed to function as adaptors between membrane proteins or with the cortical cytoskeleton (Tamura, Nei, & Kumar, 2007). Therefore, The objectives of present study were to identify PALM gene mutations by PCR-SSCP and DNA sequencing methods, evaluate the association between mutations and protein structure changes with growth and fat storage traits in two Iranian sheep breeds (Lori Bakhtiari and Zel), and the effect of mutations on Paralemmin protein structure and function.

Materials and methods

Animals

The Iranian sheep breeds including Lori-Bakhtiari (fat tail) and Zel (thin tail) were considered which originated both near the center of domestication (Biasini et al., 2014) and near the first recorded archaeological evidence of fat tail sheep (Chauhan et al., 2002). It was felt this geographic proximity would reduce false positives due to bottlenecks and selection pressure due to alternative factors such as climate, disease and pasture types. The numbers of 70 unrelated Lori-Bakhtiari from Shahrekord's research station and 70 unrelated Zel from Gorgan's research station were randomly selected. The two breeds differed in size and living conditions. Lori-Bakhtiari is a fat tail dual purpose sheep, large in size and mostly white in body color. It is a well adopted to the hilly and mountainous Bakhtiari region stretched out to southern Zagros Mountains. The Zel sheep is small in size, early maturing and mostly brown. The Zel breed is the only thin–tail Iranian breed and it is present largely on the northern slopes of the Elburz mountain range near the Caspian Sea. Blood samples were collected from each animal to measure triglyceride and cholesterol. Data of body weight at different ages measured and recorded. All 140 animals were used for genomic analysis.

Blood sample collection and DNA extraction

Blood samples (4 mL per sheep) were collected from Jugular vein and they were kept in a tube containing anticoagulant EDTA. All blood samples were placed in ice and they were transferred to the laboratory. Blood samples were stored at -20°C. The genomic DNA was extracted from white blood cells using the salting out procedure (20) and then kept at -20C. The quantity and quality of the DNA extraction was measured by Electrophoresis in 1 percent Agarose gel and Spectrophotometry method with Nano drop.

Polymerized chain reaction (PCR)

Specific primers were used for fragments amplification, by utilizing Primer3 Plus and Oligo Analyzer software's that were designed for 578 bp fragment of exon3 and 4 of PALM gene. The sequences of the primers were as follows: F: 5'-CTGGGTGAAGGTGGAGGTG-3' and R: 5'-GTTGGGAGGAGGAGGGGGTC-3'. The PCR was conducted in a 25 µl reaction mixture containing 100 ng of DNA, 2 mM MgCl2, 2 mM dNTPS, 12 pmol of each primer and 1 U of Taq DNA polymerase. The PCR product was amplified in following conditions: denaturation at 95°C in 5 min followed by 35 cycles of 95°C for 1 min for denaturation, 60°C for 1 min. for annealing primers, and extension at 72°C for 1 min. Finally, the extension at 72°C for 7 min. After PCRs for reassurance of amplification accuracy and determining extended DNA, PCR products Electrophoresis was done on 2 percent agarose gel along with 100 bp DNA marker, in 1 × TAE buffer at a fixed voltage of 80 V for 30 min. using ethidium bromide staining, the products were visualized by ultraviolet Tran's illumination.

PCR-SSCP and genotype determination

The PCR-SSCP method was applied to mutations detection within the amplified fragments. All PCR products were subjected to SSCP analysis. The aliquots of 4 µL of PCR products were mixed with 12 µL denaturing solution (99% formamide, 25 mM EDTA, 0.025% xylenecyanole, and 0.025% bromophenol blue), 10 min. denatured at 96°C and immediately they chilled on ice. For observing band patterns, we used Vertical electrophoresis tank of BioRadcompany with glass screens 18*20*0.1 cm, and 12% acrylamide gel. Samples were run for 15h at 200 V in 4°C on a 12% acrylamide: bisacrylamide gel (37.5:1). Bands were appeared by 0.1 percent silver nitrate and NaOH solution (containing 0.1 percent formaldehyde) using the protocol of Bassam, Caetano-Anollés, and Gresshoff (1991). The PCR fragments from different SSCP patterns were sequenced in both directions.

Statistical analysis

The MIXED procedure (Statistical Analysis Software [SAS], 2007) was used for the association analysis. Genotypic, allelic frequencies and Hardy–Weinberg equilibriums were estimated by GenAlEx 6.41. Traits included fat-tail weight using Domains fat tail, weaning weight, six months weight, nine months and weight at age, daily gain, and kleiber ratio are compared. The model used for the analysis was:

$$Y_{ijkl} = \mu + A_i + S_j + B_k + b(W_{ijkl} - \bar{W}) + G_{ijkl} + e_{ijkl} \quad (1)$$

In this model, Y_{ijkl}: record of each fat traits (such as back fat thickness and estimating fat tail weight) and i, j, k, l respectively, the animal level, sex, breed and Genotype; µ was the population mean; W_{ijkl}: Weight of animals at the time of blood sampling; $\bar{W}$ average weights of animals at the time of blood sampling; b regression coefficient of y on W animals; G_{ijkl} the genotype position and e_{ijkl} was the random residual effects. For calculating allelic and genotypic frequencies, we used GenALEX software (6.41 version).

Studying PALM gene evolution procedure

In this study, we used bioinformatics researches, UniportKB and NCBI databases, to investigate the Genetic variation, Gene evolution and phylogenetic of PALM gene. For human (*homo sapiens*), Mouse (*Mus musculus*), chicken (*Gallus gallus*), Cow, sheep, goat and other species, nucleotide and protein sequences of PALM gene obtained from the NCBI and Uniport database. Drawing phylogenic tree by Neighbor-Joining (NJ) method base on coding regions, sequences comparison, phylogenetic analysis and molecular evolution was performed using MEGA4 software.

Protein structure

We used bioinformatics information that include utilizing NCBI dataset, and UniportKB for retrieve the sequence of the AA of PALM gene. In this step, three-dimensional structure of protein was performed via I-TASSER and 2PHYRE software's**.** In the end, BIOEDIT 7.0 software were used for mutation detection and sequence alignments. Later, using the vectorNTI 9.0 software, with 6 open reading base was translated into protein sequence and was compared with protein sequence in UniProtKB information sources. In order to study the influence of detected mutations on protein structure and performance and also to design the three-dimensional structure of the PALM gene, we used the PHyre2 software and SWISS-MODEL section available in the EXPASY website. To evaluate the effect of identified mutations on protein structure and function of paralemmin, the website (http://blocks.fhcrc.org/sift/SIFT.html) have been used.

Results and discussion

Amplified fragments sequencing

Figure 1 displays the amplified fragments of PALM gene. Two random samples of each pattern of PALM gene (Figure 2), which have been observed in the SSCP analysis, were sent to Bioneer Company in South Korea for sequencing. Using BioEdit and Vector NT1 software, the sequences were edited and aligned. The mutations in these sequences were compared with NCBI reference sequence; Gene ID: 101109798 (*Ovis aries*). The allele frequencies and genotypic frequencies for each SNP shown in Table 1.

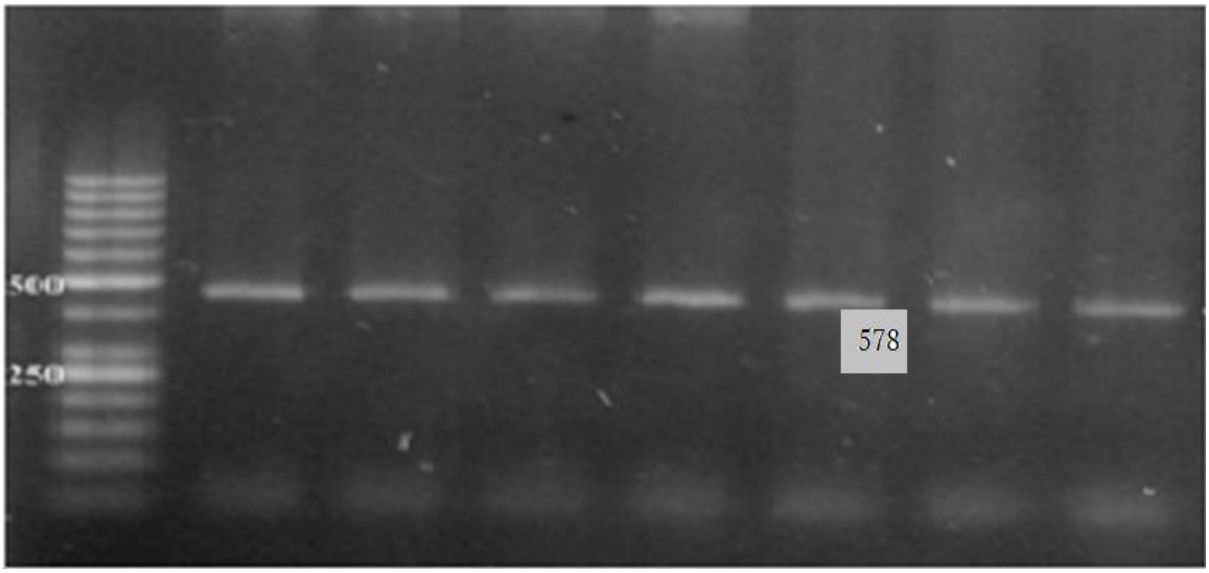


Figure 1. Electrophoresis gel of PCR products for PALM gene.

Variation in PALM gene

The nucleotide substitutions and amino acid changes in PALM gene exon 3-4 of Lori-Bakhtiari and Zel sheep breeds are shown in Figure 4. Among two identified polymorphic sites, one site was nonsynonymous substitutions and another one was Insertion mutation (49 bp). Substitution in this loci were A to G at nucleotide 7061 position. In the other words, nonsynonymous mutation causing an amino acid change of threonine to alanine. The second mutation which was insertion of a 49 bp sequence between nucleotides 6885-6886 in all SSCP patterns compared with sequences existed in the NCBI GenBank database. The results showed that the Insertion mutation in intron 3 gene PALM occurred in sheep (Figure 2).

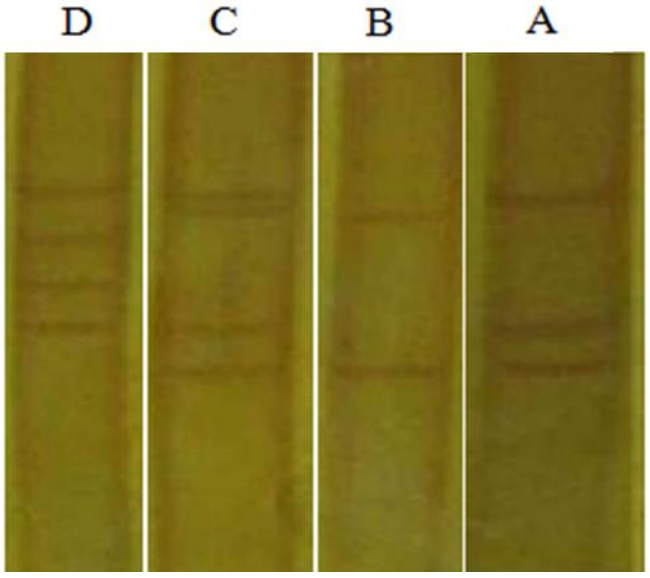


Figure 2. Band patterns on the gel for PALM exon 3-4.

Association of SNPs with growth and fat traits in Lori-Bakhtiari and Zel sheep breeds

The genotypic and allele frequencies for SNP of PALM-exon-4 & 3 in Lori-Bakhtiari and Zel breed reported at Table 1. The highest frequency of the A allele observed in the two populations. In our study, the GG mutated type of the PALM gene were not observed. The low sample size and genetic drift or existence of lethal allele G in the population could be the cause of GG genotype absence. The association between studied traits with specified genotypes are shown in Tables 2 and 3. In this study, B with A, C, D pattern were different just in one position (SNP1), with two genotypes of AA and AG.

Table 1. Genotypic frequencies and allele frequencies for SNP of PALM-exon-4 & 3 in Lori-Bakhtiari and Zel breed.

Breed	Allele G	Allele A	GG Genotype	GA Genotype	AA Genotype
Zel	0.143	0.857	0	28.6	71.4
Lori-Bakhtiari	0.064	0.936	0	12.8	87.2

Table 2. Association of genotype with growth traits in Lori-bakhtiari and Zel breed.

Traits	AA	AG
Birth weight (kg)	4.35 ± 0.24^{b}	4.75 ± 0.24^{a}
weaning weight	29.65±1.15	29.72±1.15
Daily gain (0-3) kg^{ns}	252.6±8.9	8.7±3.25
kleiber ratio (0-3) kg^{ns}	23.10±0.4	23.10±0.4

There are significant differences between AA and AG genotypes in birth weight of Lori-Bakhtiari and Zel breeds. The individuals with genotype AG had the highest birth weight. We found out, in the Lori-Bakhtiari breed there significant differences between AA and AG genotypes in cholesterol, triglycerides and fat tail weight Traits. The animals with AA genotype presented the Maximum fat tail weight and blood cholesterol, also the AG genotype has the highest triglyceride levels. However, no significant differences were observed between genotypes in Zel breed.

Table 3. Association of genotype with fat traits in Zel and Lori-bakhtiari breeds.

Breed	Traits	AA	AG
Zel	Cholesterol (%)	52.50±5.277	54.05±5.277
	Triglyceride (%)	15.30±3.932	15.62±4.872
Lori-bakhtiari	Cholesterol (%) **	65.57 ± 2.625^{a}	58.57 ± 2.625^{b}
	Triglyceride (%) *	33.15 ± 2.561^{b}	37.01 ± 0.829^{a}
	Fattail-W(kg) **	4.87 ± 0.23^{a}	4.6 ± 0.23^{b}

Evolution procedure results

The phylogenetic results showed that, the phylogenetic tree is rooted and have common ancestor. Likewise, PALM gene, according to evolutionary path, is classified into two separate categories. In first covey, *Gallus gallus* and in second one, other species in several branches, so that the sequence of cow and sheep is placed in a sub-branch which forms a clade beside goat. However, a sensible distance between sequences of *G. gallus* and other animals can be seen. The comparison of illustrated coding region sequences, this gene among different species, is of orthologous genes which are derived from a common ancestor (Figure 3).

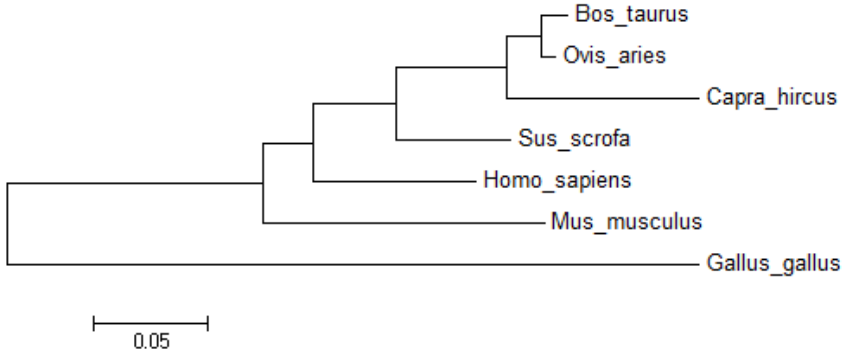


Figure 3. Phylogenetic tree of PALM gene sequences drawn using Clustal W and MEGA6.

The effect of mutations on paralemmin protein structure and performance

Amino acid sequence analysis often provides important insights into the tertiary structure and biological function of proteins. The basic strategy is first to find the similarity of sequences in the forms of pairwise sequence alignments, multiple sequence alignments and homology searches against the database, and then to infer 3-D structural similarity and/or functional similarity. The prediction of protein tertiary structure using sheep PALM amino acid sequence is shown in Figure 2. The structure of the residues 282 (73 percent of sequences), to confidence 92.2 percent in the highest scale similar crystal structure of the protein chain Atropomyosin (c1c1gA _ 92.2 percent) has been modeled.

Chasman and Adams (2001), obtained their best prediction results when the query protein was at least 60 percent identical to its homology. The tertiary protein structure was predicted Similar to proteins string kind of contractile proteins. The main unit structure in this helixes model that are connected by a cross connections, can form strong and insoluble in water conservation structures. In other words, the tropomyosin, a string protein (contractile protein), often have a structural role. In other hand, the paralemmin, the active sites plasma membrane filopodia tips and microspikes and also a role for this protein in plasma membrane dynamics and in the control of cell shape (Kutzleb et al., 1998), expressed in the lens fiber cell maturation and forming the cytoskeleton cortical (Chauhan et al., 2002; Bagchi, Katar, Lo, & Maisel, 2003). With the formation and/or stabilization paralemmin 1 in the plasma membrane forms a filament contractile proteins are aligned. Non-synonymous signal nucleotide polymorphisms are coding variants that introduce amino acid changes in their corresponding proteins. Interestingly, nsSNP can affect protein function, they are believed to have the largest impact on human health compared with SNPs in other regions of the genome (Chauhan et al., 2002).

As mentioned above, a point mutation of A to G at nucleotide 7061 that causes the amino acid threonine to alanine. Transversion of PALM gene occurred in exons 3 and 4. Expected amino acid substitution on protein function can have a significant impact on the sheep species. Here we provided the amino acid substitution and prediction methods (SIFT), which uses sequence and/or structure to predict the effect of an amino acid substitution on protein function. The SIFT method was applied to find nsSNPs. The alignment protein sequence of the gene was identified in most species, paralemmin gene at codon 60 protein sequences in poultry, there is the amino acid alanine.

Figure 4 displays the substitution of the codon 60 amino acid alanine tolerated and often this change does not affect the biological activity of proteins, because in most proteins, the amino acid can undergo changes along with at least a significant impact on protein activity without the cells can tolerate. However, the interaction with other amino acids revealed that the threonine neutral and polar and nonpolar neutral amino acid such as alanine is mutated, the interaction with the negatively charged glutamate, may have enough power to keep fold the protein in its natural state, therefore, may not be able to perform its cellular function. As it shows in Figure 5, the fold of protein with alanine substitution to change that this protein can impact on performance.

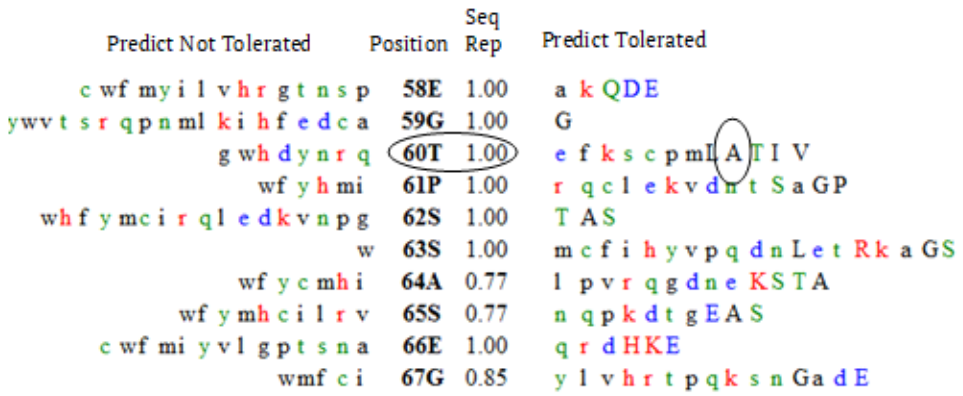


Figure 4. Threshold for intolerance is 0.05. Amino acid color code: nonpolar, uncharged polar, basic, acidic. Capital letters indicate amino acids appearing in the alignment, lower case letters resulted from prediction. 'Seq Rep' is the fraction of sequences that contain one of the basic amino acids. A low fraction indicates the position is either severely gapped or unalignable and has little information. Expect poor prediction at these positions.

The second insertion mutation of the 49 bp sequence between nucleotides 6885-6886 in all SSCP patterns compared with existed sequences in the NCBI database (Figure 6). GENSCAN program was applied to the

problem of gene identification, which identifies complete exon/intron structures of genes in genomic DNA (Burge & Karlin, 1997). Finally, to ensure accurate location at Insertion mutations and its impact structure gene and protein function, Assemble are sequence inserted (49 bp) with vector NTI 9.0 software with the NCBI reference sequence and software GENSCAN predicted exons of the gene was discussed. The results showed that the Insertion mutation occurred in intron 3 of the Paralemmin gene in sheep and the fourth exon of the 7153-7023 to 7072-7202 nucleotides, is a displacer which contrasts reports in the fourth exon of this gene in sheep Paralemmin Ensembel database (Figure 6). The fourth exon probability of 99 percent is expected to be to design primers, cDNA amplification or other purposes can be used (Figure 7).

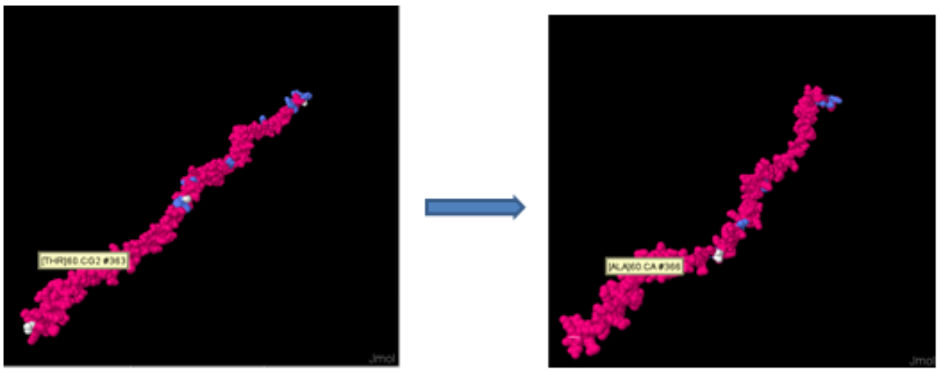


Figure 5. Predicted Paralemmin Protein 3rd structure (Jmol is a point mutation in 60 codon in substituting Thereonine with Alanine. It changes Paralemmin protein folding).

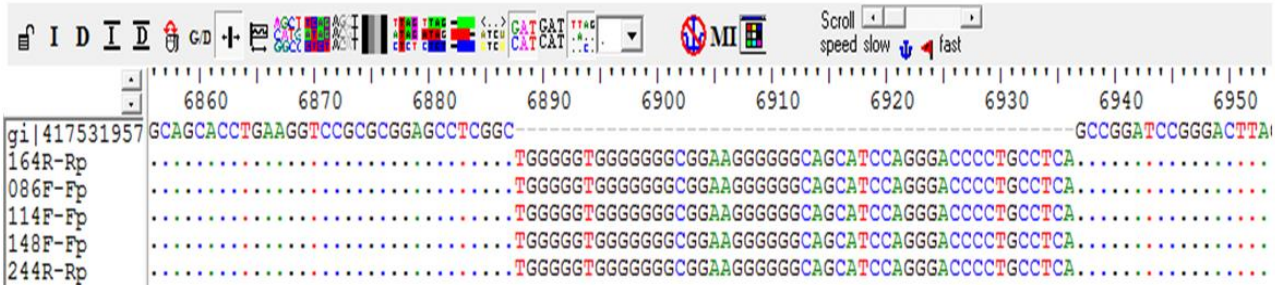
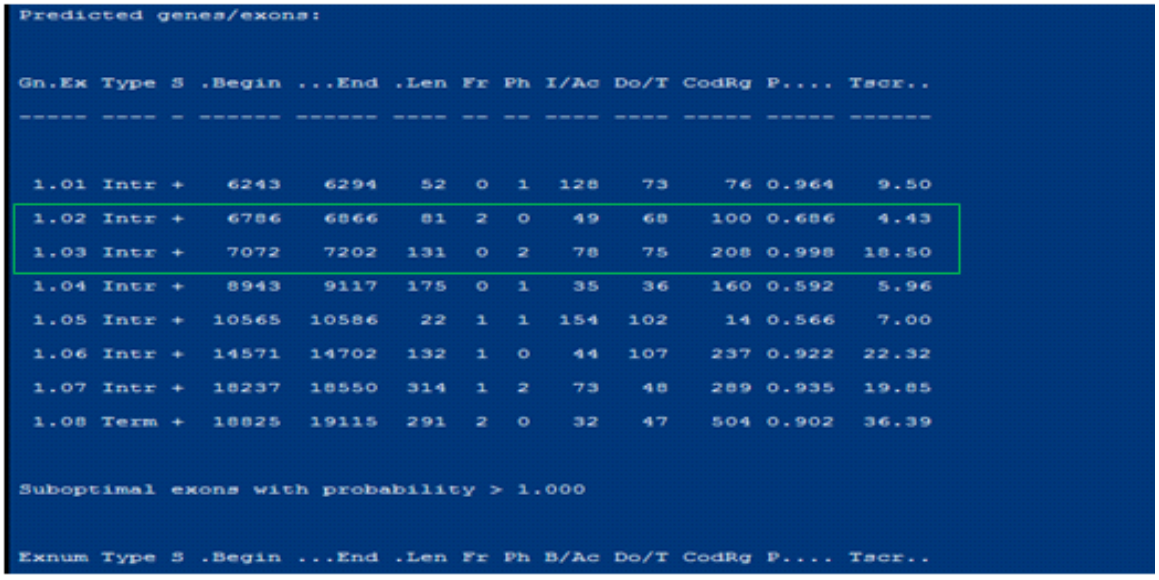


Figure 6. The results of simulated rows of unique samples of SSCP (164-114-148-244-086) and PALM1 of *Ovis aries* in NCBI GeneBAnk database with BioEdit software with 6686-6885 of PALM 1 gene from NCBI (NC_09462.1) a mutation has occurred in G 49 bp that is considered as intron3 gene with predicting gene.



Predicted genes/exons:

Gn.Ex	Type	S	.Begin	...End	.Len	Fr	Ph	I/Ac	Do/T	CodRg	P....	Tscr..
1.01	Intr	+	6243	6294	52	0	1	128	73	76	0.964	9.50
1.02	Intr	+	6786	6866	81	2	0	49	68	100	0.686	4.43
1.03	Intr	+	7072	7202	131	0	2	78	75	208	0.998	18.50
1.04	Intr	+	8943	9117	175	0	1	35	36	160	0.592	5.96
1.05	Intr	+	10565	10586	22	1	1	154	102	14	0.566	7.00
1.06	Intr	+	14571	14702	132	1	0	44	107	237	0.922	22.32
1.07	Intr	+	18237	18550	314	1	2	73	48	289	0.935	19.85
1.08	Term	+	18825	19115	291	2	0	32	47	504	0.902	36.39

Suboptimal exons with probability > 1.000

Exnum	Type	S	.Begin	...End	.Len	Fr	Ph	B/Ac	Do/T	CodRg	P....	Tscr..

Figure 7. GENSCAN results that can predict internal and terminal exons. The specified region of exon no. 3 and 4 of Paralemmin gene is tracked as identified mutations in this study. GnExType: gene no. and exon, prediction type, exon or signal (S, A) of predicted strands. End, Begin: End point or begin point, Le: predicted exon length, Fr: base frame reading, P: probability.

Conclusion

In present survey we identified two polymorphic sites including a nonsynonymous substitution and an insertion mutation (49 bp). Substitution in this loci were A to G at nucleotide 7061 position. The second mutation was insertion of a 49 bp sequence between nucleotides 6885-6886. Non-synonymous mutation deduced Thr20Ala amino acid exchange and ensuing two different structures for paralemmin protein that could be potentially affect protein structure and function during the interaction with glutamate in the cytosolic surface of plasma membrane. PALM gene, according to evolutionary path, is classified into two separate categories. In the first one, *G. gallus* and in the second one, other species in several branches, so that the sequence of cow and sheep is placed in a sub-branch which forms a clade beside goat. Comparison of illustrated coding region sequences of PALM gene among different species revealed that this gene is of orthologous which are derived from a common ancestor.

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