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An In/Del mutation in upstream regulatory region of MC1R gene associated with grey lethal disease in grey Shiraz sheep (Persian lamb)

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ABSTRACT

In the present study, MC1R, which play an important role in normal pigmentation in Skeen and wool, was candidate to assess the lethal grey disease in Persian lamb. Blood samples (50) were collected randomly from grey Shiraz sheep, and DNA was extracted by salting out method. One of these samples showing the disease was assigned as control case. Two pairs of specific primers of P1MC1R and P2MC1R were designed to amplify two fragments from upstream regulatory region (URR) and coding sequence (CDS) of MC1R gene. After genotyping by SSCP technique, samples from each banding patterns were sequenced and analyzed using BioEdit and DNASIS MAX softwares. Comparing sequences from control (sick lamb) with healthy ones showed different haplotype in products of each specific primer pairs. The multiple alignments revealed a 26 bp In/Del occurring at PCR product of P1MC1R gene in control case which was not observed in other studied lambs, and also 7 and 11 different positions were seen between sequence amplified by P1MC1R and P2MC1R primers, respectively. Further bioinformatics analysis showed that 26 bp insertion/deletion (In/Del) occurred in control case P1MC1R sequence caused deletion of gamma_IRE_CS and LBP_1_RS motifs from URR of control case. It seems this 26 bp In/Del mutation might have changed its expression and can be potential cause of grey lethal disease in studied sheep breed.

Key words: Grey lethal disease, Grey Shiraz sheep, MC1R gene, Mutation, Sequencing

The grey Shiraz sheep (Kaboudeh Shiraz, Persian lamb) is one of the world's skin breeds which its one-day lamb fur is used for preparation of expensive clothing and hats. Also, because of its blue sky colour, it is named Kaboudeh Shiraz or grey Karakul. This breed is reared mainly in Fars province, south west of Iran. Due to the expanse of the province and climatic variation, this province has always been considered as one of the major regions of sheep farming in Iran. Almost 6 million sheep and lambs are reared in Fars province, which estimates that more than 1.5 million sheep and lamb are from the grey Shiraz breed. The mean birth and 3 months (weaning) weights of Persian lambs for male and female lambs are 4.4, 3.9 and 20.96, 19.8 kg, respectively. Also, the means wool weights of ewes and rams are 1.3 and 2.2 kg, respectively (Karimzadeh *et al.* 2015). Coat colour in animals does not only play an aesthetic role, but also has substantial impact on many factors indispensable for survival. Some of the identified coat colour controlling genes shows pleiotropic effect while also affecting behaviour and disorders, often of lethal character (Charon and Lipka 2015). Most of these diseases have their counterparts in humans (Reissmann and Ludwig 2013).There are also evidences that coat colour genes influence production and reproduction traits (Becerril *et al.* 1993, Johansson *et al.* 2005), having an impact on economic effects of animal breeding.

Several genes are involved in the formation of pigment in the skin and hair of many vertebrate which are common among the various forms of species (Rieder *et al.* 2001). These genes considered as genes for making melanin pigments to form coloured pigments by melanocytes cells (cells in the junction of dermis and epidermis layers of the skin).The melanocortin 1 receptor (MC1R) and agouti-signaling-protein (ASIP) genes are from these genes that were coded by extension (E) and agouti (A) regions and control the melanin pigments in mammals (Rieder *et al.* 2001).The MC1R gene is active in melanocytes and controls the amount of eumelanin production. It is located in locus extension, over 950 base pairs long, has only one exon, but shows high polymorphism (Scherer and Kumar 2010).The MC1R gene is known as a strong marker to formation of

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melanin. Research on genetic diversity of candidate genes affecting human body colour in different populations and high levels of polymorphism in the MC1R locus have shown that over 30 different alleles have been identified in this gene (Sturm *et al.* 2001).

There are seven postulated coat colour loci in sheep which are described (number of alleles in parentheses), and their main functions are A(12): colour patterns. This locus regulate patterns of tan phaeomelanin (or white) and black or moorit (chocolate brown) eumelanin; B(2): pigment types. Main effect of this locus is determined by black or moorit eumelanin pigment; C(2): full pigmentation or albinism; E(2): presence or absence of dominant black; G(2): presence or absence of agouti banding (Sur); S(2): presence or absence of white markings; W(3): presence or absence of dominant (Persian) white and dominant (lethal) grey (Adalsteinson 1983).The black, grey, white and brown are predominant coat colours in Karakul pelts. The intensity of grey colour varies considerably depending on the ratio of black (brown)/white fibers in the pelt. It was shown that heritability of the intensity of grey colour is high (0.35 to 0.7) (Adalsteinson 1983, Nel 1966).

In Karakul, the white is not inherent, but was introduced by crosses between black Karakul and white wool Persian (Nel 1966). The white color allele (W) is partially dominant to black and brown allele (B and E). Homozygous WW ($A^{wh}A^{wh}BBE^{D}E^{D}SSWW$) lambs are pure white and heterozygous Ww lambs show white body color with black or brown face and sometimes small spot on body (white C- or white B-). Moreover, it has been shown that white karakul (WW) is similar to grey karakul, but after weaning, white lambs live more than grey ones (Schoeman 1998).

There is a strong association between MC1R genotype and phenotype in vertebrates due to polymorphisms in this gene and diversity of produced melanin. Also because of low negative pleiotropic effects of polymorphisms in this gene, as a good candidate gene, the MC1R alleles have been introduced to determine the clarity of the skin and its effective alleles determine the darkness of body cover. While, it is recessive and ineffective alleles show a brighter colour (Loher *et al.* 2008). Mahmoud *et al.* (2017) showed that the two independent non synonymous Met73Lys and Asp121Asn mutations in MC1R gene are associated with black or red coat colours in sheep breeds. Also, Kinikci *et al.* (2016) by analyzing of goat MC1R sequences with the PAML 4 software provided evidence that two SNPs (c.764G>A and c.801C>G) in this gene might evolve under positive selection. Therefore, due to high polymorphic behaviour of MC1R gene and its association with body colour, the aim of the present study was to investigate MC1R polymorphisms in URR and coding sequence using molecular (SSCP assay and sequencing) and bioinformatics analysis and its association with lethal grey disease in grey Shiraz sheep.

MATERIALS AND METHODS

Sampling and DNA extraction: Blood samples (50) were collected randomly from grey Shiraz sheep with different body colour. There was a 3-month-old light grey and sick lamb probably carrying grey lethal recessive allele. This lamb selected as control case had larger rumen and frail appearance that was consistent with the symptoms of grey lethal disease (Groenewald1992). The blood samples were collected in EDTA treated pipe and stored at –20°C until further analysis. The DNA was extracted using modified salting out method (Miller *et al.* 1988) and its quality and quantity were measured via agarose gel electrophoresis and spectrophotometry, respectively.

Primer designing and polymerase chain reaction: To amplify 2 segments from URR and CDS of MC1R gene, 2 specific primer pairs (P1MC1R and P2MC1R) were designed by Oligo7 software according to reference sequence (NCBI: Z31369.1) (Table 1). The PCR reaction mixture was prepared in 25µl final volume containing 0.1 mM of each dNTPs, 10 pM of each primer, 2.5 µl of 1× reaction buffer, 1.5 mM $MgCl_2$, 0.05 U *Taq* DNA polymerase, and 100 ng of template DNA. PCR thermal cycling profile consisted of an initial denaturing step at 94°C for 5 min, followed by 30 cycles of 45 sec at 94°C, 45 sec at 65–67°C (primer annealing), 45 sec at 72°C, and a final extension step of 10 min at 72°C. Presence of PCR product was tested on a 1% agarose gel.

SSCP assay: Single strand conformation polymorphism (SSCP) assay was used to detect banding patterns of amplified fragments. For this purpose, after denaturation at 96°C for 5 min in presence of formamide, the amplified fragments were electrophoresed on 8% polyacrylamide gel. After electrophoresis, the gels were stained with silver nitrate.

Sequencing and bioinformatics analysis: Further verification of detected alleles was done by sequencing the samples (1 to 3 samples per banding patterns) using the ABI 3730XL DNA Analyzer (Bioneer Corporation, Daejeon, Korea, Republic of (South Korea). Before sequencing, PCR products were purified using DNA purification kit from Roche Company according its instructions. Obtained sequences were multiple aligned by BioEdit software to finding base pairs differences and also

Table 1. Sequences of designed primer pairs in present study

Locus	Primer	Sequence (5'–3')	Position	Product length (bp)
MC1R	P_1MC1R	F: CTGCAGGTCCTTCTGGACTCR: CATTGCTCCTCTTCTGAGCA	URR*	379
	P_2MC1R	F: ACTCCTCATTCCACCTGCACR: GCAGATCATGGAGTCGAACA	CDS**	365

*Upstream regulatory region, **Coding sequence

different DNA motif sequences were identified using DNASIS MAX software.

RESULTS AND DISCUSSION

Two fragments with lengths of 379 and 365 bp from Grey Shiraz Sheep MC1R gene were amplified successfully by spefically designed primer pairs. The sick sample (carrying grey lethal gene) marked by number 78 was considered as control. The PCR products were genotyped by SSCP technique and after determining different banding pattern for each primer pairs, some samples of each pattern were selected randomly for sequencing.

In URR site (P1MC1R) that is shown by A for ease, the PCR products of samples A78 (grey, control), A22 (brown), A57 (brown) and A122 (black) were sequenced. The sequence of A122 sample was not appropriate and hence was removed from the analysis. In A78 (control), a 26 bp deletion was observed (Fig. 1). This deleted segment existed and was same in both A22 and A57. Therefore, it seems that an In/Del mutation occurred in URR sequence of P1MC1R. There might be an indication that this deletion in A78 sample sequence caused loss of grey lambs at the age of 3–4 months. Also, a deletion in grey-lethal gene induces severe malignant autosomal recessive osteoporosis in mouse (Chalhoub *et al.* 2003). Rieder *et al.* (2001) compared a 1721 bp region of MC1R gene between 11 different body colour phenotype in horse and found an 11 bp deletion in exon 2 of this gene. Also, in present study, investigating the sequence differences showed an insertion mutation of a thymidine (T) at position 49 of A22 sample, which did not existed in other samples (Fig. 1). It is now known that the gene that causes the grey colour is sub-lethal when homozygous, which explains why grey to grey mating of Karakul sheep is avoided (Lundie 2011). The sub-lethal gene, lethal roan, is present in the Karakul breed known in Russia as Shirazi, important in the production of grey pelts for the fur trade (Muhaghegh-Dolatabady and Habibizad 2014).

Further bioinformatics analysis showed that 26 bp In/Del occurred in control case P1MC1R sequence caused deletion of gamma_IRE_CS and LBP_1_RS motifs from URR of P1MC1R gene. Also, the GMCSF_CS and BHLH_CS motifs were observed only in control and A57 samples, respectively. It seems that 26 bp In/Del mutation occurred in P1MC1R products might have changed its expression and can be potential cause of grey lethal disease in studied sheep breed. Eukaryotes promoter contain multiple regulatory motifs that are necessary to control gene

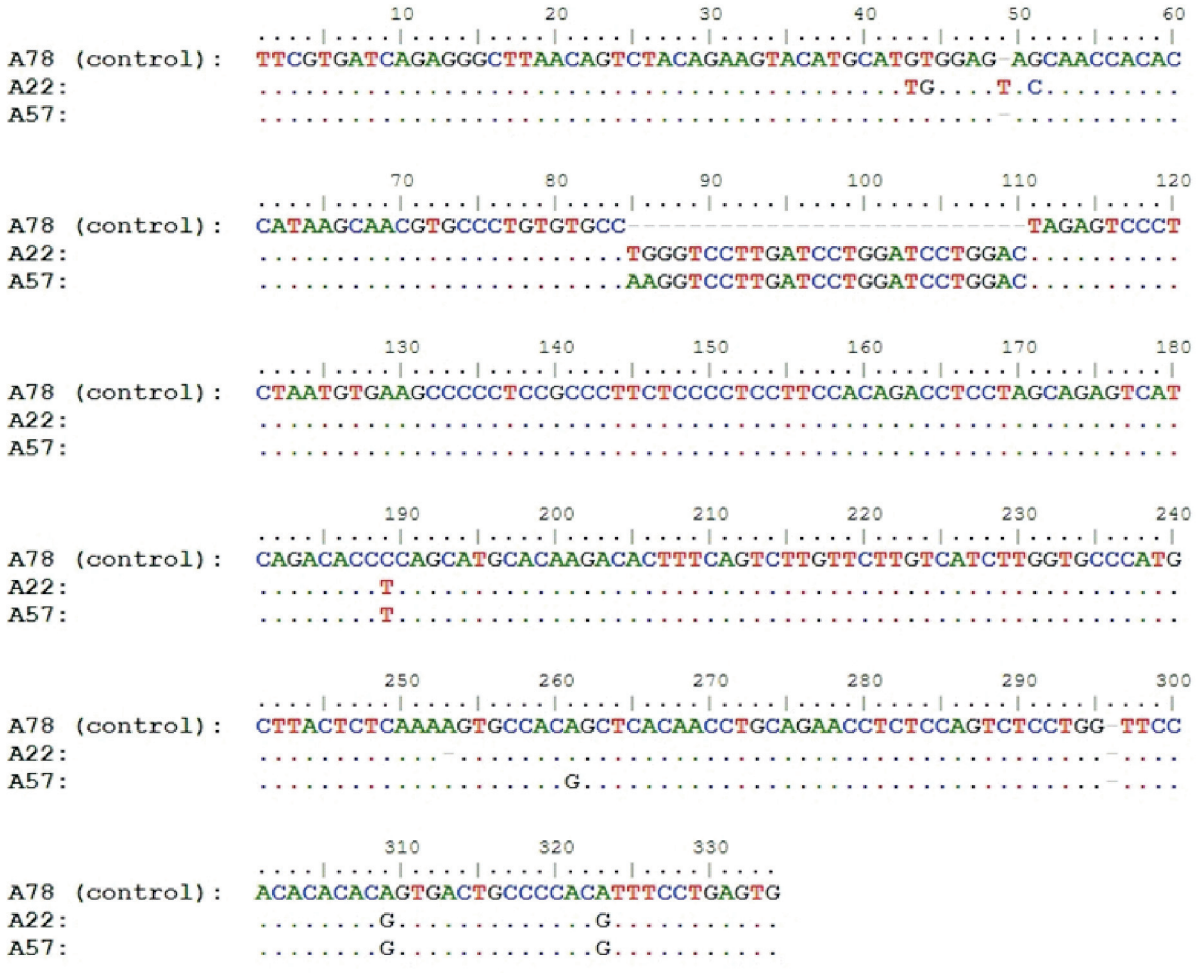

Fig.1. Alignment of P1MC1R sequences from three samples (A78, A22 and A57).

Table 2. Sequence differences in URR of MC1R in studied lambs

Sample	Position on sequence (bp)									
	43	44	49	51	85-110***	189	253	261	309	323
A78*	G	T	-**	G	-	C	A	A	A	A
A22	T	G	T	C	TGGGTCCTTGATCCTGGATCCTGGAC	T	-	A	G	G
A57	G	T	-	G	TGGGTCCTTGATCCTGGATCCTGGAC	T	A	G	G	G

*Control sample; **Deletion; ***the 26 bp In/Del site.

Table 3. Motifs available at genotyped sequences using DNASIS MAX software

Motif name	Motif pattern	Studied sample	Start (bp)	End (bp)	Motif name	Motif pattern	Studied sample	Start (bp)	End (bp)
AP_2_CS6	CCCMNSSS	Control	106	113	GMCSF_CS	CATTW	Control	294	298
		A22	133	140			A22	N	N
		A57	132	139			A57	N	N
bHLH_CS	CANNTG	Control	40	45	gamma_IRE_CS	CWKKANNY	Control	N	N
		A22	N	N			A22	91	98
		A57	40	45			A22	98	105
CAP_site	CANYYY	Control	21	26			A57	90	97
		Control	158	163			A57	97	104
		Control	177	182	HIS4_US	TGACT	Control	283	287
		Control	183	188			A22	309	313
		Control	198	203			A57	309	313
		Control	233	238	LBP_1_RS	WCTRG	Control	N	N
		Control	240	245			A22	109	113
		Control	258	263			A57	108	112
		Control	294	299	NRE_Box1_CS	ANCCTCTCY	Control	250	258
		A22	21	26			A22	276	284
		A22	185	190			A57	276	284
		A22	204	209	TCF_1_CS	MAMAG	Control	19	23
		A22	210	215			Control	131	135
		A22	225	230			Control	223	227
		A22	259	264			Control	231	235
		A22	266	271			Control	278	282
		A22	284	289			A22	19	23
		A57	21	26			A22	158	162
		A57	184	189			A22	249	253
		A57	203	208			A22	257	261
		A57	209	214			A57	19	23
		A57	224	229			A57	257	161
		A57	266	271			A57	249	253
		A57	284	289					
EARLY_SEQ1	YYCCGCCC	Control	109	116					
		A22	136	143					
		A57	132	139					

N, Non common motifs in different banding patterns.

expression, so comparing frequency and distribution pattern of the motifs among different species and breeds is an effective approach for understanding its evolution in vertebrates. A motif is short sequences of DNA with repeated patterns that are biologically functional and most of them are specific binding sites for proteins such as nucleases and transcription factors. Some of them are also involved in important processes at RNA level such as ribosome binding, processing of mRNA (splicing, editing, polyadenylation) and transcription termination (D'haeseleer 2006). Therefore, the potential role of motifs that were found in present study can be further investigated as a functional mutation. The motifs along with their positions are listed in Table 3.

In addition, other observed differences between 3 sequences are indicated in Fig. 1. At positions of 189, 309 and 323 bp, the A22 and A57 were same but both different from A78 (Table 2). According to obtained results, there were three different haplotypes in P1MC1R sequence, so that each sequence from each samples represented a separate

Table 4. Sequence differences in coding sequence of MC1R in studied lambs

Sample	Position on sequence (bp)									
	27	28	61	80	100	123	179	186	237	273
B78*	G	C	G	-**	G	-	C	-	C	C
B2	G	C	C	G	A	-	G	-	C	C
B49	C	G	C	G	A	A	G	C	G	T
B65	G	C	G	-	G	-	C	-	C	C
B1	G	C	G	-	G	-	G	-	C	T
B100	G	C	G	-	G	-	C	-	C	C
B122	G	C	C	G	A	-	G	-	C	C

*Control sample; **Deletion.

haplotype.

In the P2MC1R locus that for ease represented by B, seven samples, viz. B78 (grey), B2 (grey), B49 (brown), B65 (grey), B1 (grey), B100 (sur) and B122 (black) were amplified and sequenced. In this locus, the sequence of B78 as control was compared with other samples. The observed differences between control and other as well as between other sequences are presented in Fig. 2. Comparing the sequence of studied samples with control revealed different base substitution differences except for B65 (Fig. 2).

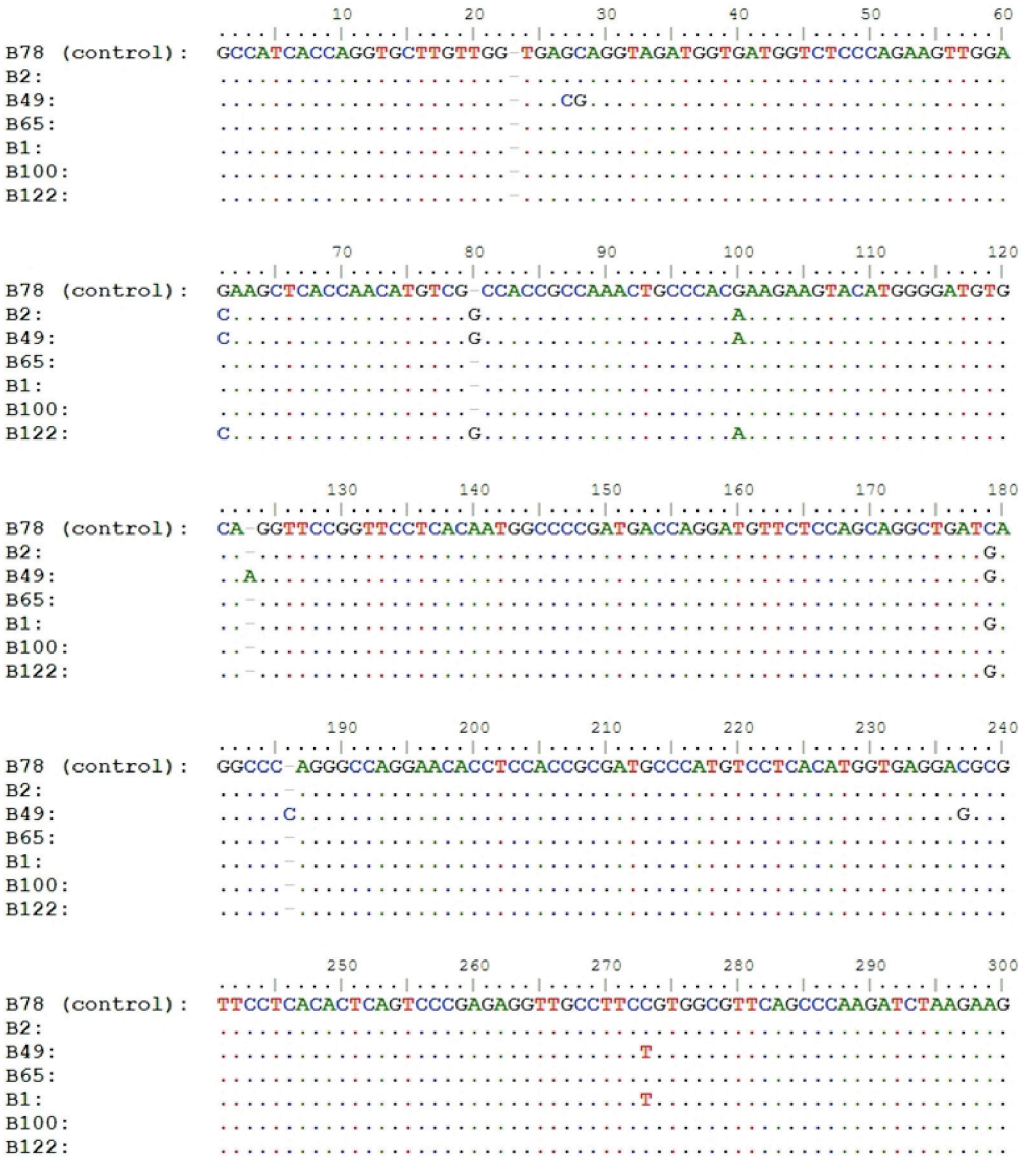

Fig. 2. Alignment of P2MC1R sequences from seven samples (B78, B2, B49, B65, B1, B100 and B122).

According to these results, four haplotypes, viz. (B78, B65 and B100), (B2 and B122), B49 and B1 were detected in P2MC1R locus (Table 4).Yang *et al.* (2013) sequenced the complete coding region and parts of the 52 - and 32 - untranslated regions of the MC1R gene in Chinese sheep and showed 5 SNPs (2 non-synonymous mutations previously associated with coat colour and 3 synonymous mutations). They were suggested that the mutations of MC1R gene are associated with black coat color phenotype in Chinese sheep.

In conclusion, there was a 26 bp deletion in PCR product of URR region of MC1R (P1MC1R) locus (sample 78, control) which was not observed in other samples sequences. Therefore, it is likely this In/Del mutation is the main cause of grey lethal disease in Persian lamb. Gene expression studies could help unravel the association between 26 bp In/Del mutation in the MC1R promoter and risk of lethality in grey colour of Karakul sheep.

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