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Polymorphism of *IGF-I* and *ADRB3* Genes and Their Association with Growth Traits in the Iranian Baluchi Sheep

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ABSTRACT

Baluchi sheep is considered as the most common breed in Iran, constituting about 30% of total sheep population, approximately 15 million heads. This research was designed to study the incidence of mutation in two loci of *IGF-I* (Exon 3) and *ADRB3* (Intron) along with their association with body weight traits in Baluchi sheep population. Following DNA extraction from 190 Baluchi sheep, two pairs of primers were designed to amplify each gene. PCR-SSCP (Single-Strand Conformation Polymorphism) and DNA sequencing were employed to detect polymorphism of the mentioned genes. Two banding patterns were detected for *IGF-I* locus. The frequencies of AA, AB were recorded as 0.89, 0.11, respectively in *IGF-I* locus. As for *ADRB3*, two patterns corresponding with two genotypes (their frequencies mentioned in parentheses) of AA (0.85) and AB (0.15) were identified. One SNP change was observed in *ADRB3*, and one in *IGF-I*. A previously reported SNP was detected in exon 3 of *IGF-I*. The effects of *IGF-I* and *ADRB3* polymorphism on the corrected phenotypes for body weight at birth (BW), weaning (WW), 6 months of age (6MW), 9 months of age (9MW) and 12 months of age or Yearling Weight (YW) were examined using least square methods. No significant association was detected between the polymorphism of *IGF-I* and body weights. As for *ADRB3*, the genotype AA was found out to exert a significant positive effect on 6MW (AA, 30.20±1.85 kg day^{-1}; AB, 27.67±1.98 kg day^{-1}; P<0.05).

Keywords: Baluchi sheep, β3-Adrenergic Receptors (ADRB3), Insulin-like Growth Factor (IGF-I), PCR-SSCP, Polymorphism.

INTRODUCTION

There exist about 27 sheep breeds in Iran, which vary in their genetic potential for production of meat, milk, wool and pelt; disease resistance; fecundity (Tavakolian, 2000). Iranian sheep population amounts to about 54 millions. Sheep is considered as the country's most important domestic animal mainly used to provide meat. There are varieties of different indigenous sheep in different locations of Iran without a proper definition of their distinct breeds. However, they are considered as geographically defined populations (Molaee *et al.,* 2009). Also (in Iran) lamb meat and mutton are traditional sources of protein with their consumption levels being high in comparison with meat from cattle and goats (Tahmoorespur *et al.,* 2009). Due to the fact that the supply of meat from sheep does not adequately meat the demand, a breeding scheme is needed to increase the efficiency in sheep production through an improvement litter size, body conformation, lamb weight and ewe milk yield (Yazdi *et al.,* 1997). Profitability of sheep keeping for meat

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production depends to a great extent on lamb weight, so the selection objective is to concentrate on theis determining trait (Tosh and Kemp, 1994).

Bluchi sheep is the most common native breed of sheep in Iran, comprising 30% of the sheep population. This breed is native to the eastern parts of the country and as well widespread in the southern parts of Khorasan, Sistan and Balouchestan, Yazd and Kerman Provinces which suffer from dry and hot climates. The breed is fat-tailed with white fleece spots of black markings on their head and legs. This breed is primarily employed in meat production with the wool very suitable to be used in wearing of carpets (Abbasi *et al.,* 2011; Hosseinpour Mashhadi *et al.,* 2005).

For the past forty years, animal breeding and genetic improvement in Iran and as well in the other parts of the world have been made possible by selection based on phenotypic information. This is a far back method through which it is very difficult to make fast genetic improvements. However, the genetic improvement in some polygenic traits like growth and other production traits, can be enhanced through Marker or Gene-Assisted Selection (MAS or GAS) which benefits from a higher accuracy in estimating the genetic value of animals (Dekkers, 2004; Ranjbari *et al.,* 2012). Candidate gene strategy, a main approach, is utilized to identify genetic variation in genes affecting the physiological pathways related to a phenotype, which would be more likely to affect the quantitative variation in that phenotype than genes or chromosome regions chosen as by chance (Muhaghegh-Dolatabady *et al.,* 2012; Schwerin *et al.,* 1995).

In animal industry, growth traits of the animal are always of primary concern during breeding for their determining economical value (Zhang *et al.,* 2005). Body weight traits, despite being quantitative in nature along with some of their such major genes as Insulin-like Growth Factor I (IGF-I) and β3-Adrenergic Receptors (ADRB3) have so far been identified (Forrest and Hickford, 2000; Forrest *et al.,* 2006; Forrest *et al.,* 2007; Horrell *et al.,* 2009; Yilmaz *et al.,* 2005; Zhang *et al.,* 2008).

Insulin-like Growth Factor I (IGF-I), its their important role in growth regulation, development and metabolism in mammals has been chosen as a candidate gene in this study. Another key gene related to the growth rate is *ADRB3*. *ADRB3* belongs to the R7G superfamily of G-protein coupled receptors playing pivotal role in the regulation of energy metabolism in mammals. It is predominantly found on the surface of adipocytes. In sheep, intronic polymorphism of the *ADRB3* gene has been reported to be associated with birth weight, growth rate, carcass composition, cold survival as well as lamb survival (Forrest *et al.,* 2003).

In spite of the functional importance of *IGF-I* and *ADRB3* on growth traits, no reports have been provided on the effects of the *IGF-I* and *ADRB3* genes polymorphism on growth traits in Baluchi sheep. Therefore, the objectives followed in the present study were to identify polymorphisms of Insulin-like Growth Factor I (IGF-I) along with β3-Adrenergic Receptor (ADRB3) genes by means of PCR-SSCP and sequencing, and as well to investigate their association with growth traits in Baluchi Sheep.

MATERIALS AND METHODS

Sample Collection and DNA Isolation

Venous jugular blood samples (5 ml per sheep) were randomly collected from 200 Baluchi sheep using vacuum tubes treated with 0.25% Ethylene Diamine Tetracetic Acid (EDTA), in Animal Breeding Center of North-East of Iran, immediately placed on ice and transferred to the laboratory in a Thermos flask, within 2 hours. The collected blood samples were further transferred to the laboratory using cooling chain and stored at -20^{o}C for further analysis. Genomic DNA was extracted from whole blood samples by means of a

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commercially available kit (Diatom of Isogene Lab. Ltd Co., Moscow, Russia). Following a measurement of the DNA concentration and a determination of its purity (quality and quantity) through spectrophotometry and agarose gel electrophoreses, the tubes containing the isolated DNA were diluted to a final concentration of 50 ng μL^{-1} in sterile distilled water, stored at 4°C and later used in the subsequent PCR reactions.

Primers for PCR Amplification

Specific primers (Table 1) were designed to amplify one region of *IGF-I* gene (Exon 3) corresponding to the GenBank accession number X69473.1 and one region of *ABDR3* gene (Intron) corresponding to accession number AF109928. The primers were designed using the Primer Premier 5.0 software (http://www.premierbiosoft.com/). This software produced many sets of primers, all of which were entered into the BLAST algorithm to search for homology between the sequences and other genes in GenBank. Those primers showing homology with genes other than the mentioned ones were discarded.

PCR Amplification

Polymerase Chain Reaction (PCR) was carried out using ~50 ng of genomic DNA, 0.5 µM of each primer, 2.5 µL 10x buffer with 1.5 mM $MgCl_2$, 150 µM of dNTPs and 1.5 unit of *Taq* DNA polymerase (Ferments. USA). The final volume was 25 µL. Amplification was carried out under the following conditions: (i) 5 minutes of denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 30s; (ii) annealing at a temperature of the primer used (mostly around 53-56°C) for 30s and iii) extension for 45 seconds at 72°C, with a final extension of 72°C for 10 minutes. PCR products were detected through electrophoresis on 2% agarose gels. Gels were stained with ethidium bromide.

PCR products were also resolved through SSCP, with the aim of detecting any polymorphism occurring within the sequence. The results obtained after SSCP analysis were confirmed by sequencing 20 randomly chosen DNA samples for each primer pair, in both forward and reverse directions. All sequences from two loci were checked with their GenBank accession numbers.

SSCP Condition for the Mentioned Genes

PCR products were resolved through SSCP analysis. The amplified fragments (8 ml) were mixed with an equal volume of sample buffer [95% of Formamide, 20 mM EDTA (pH 8.0), 0.05% Xylene Cyanol, and 0.05% Bromophenol blue]. A 10% SSCP gel mixture (30 ml) was prepared through acrylamide:bisacrylamide (49:1), TEMED (30 µL), and 10% ammonium persulfate (0.8 ml) in a lx TBE (90 mM Tris-borate at pH 8.3, 4 mM EDTA) and a voltage of 300V, running time (8 hours) and running temperature of 4ºC. Each PCR reaction was diluted in denaturing solution, denatured at 95ºC for 5 minutes, chilled on ice and resolved on polyacrylamide gel. Electrophoresis was carried out in a vertical

Table 1. Sequences of primer used in PCR amplification.

Gene	Primer sequence (5'-3')	Amplicon size	Location	Accession number
IGF-I	Fw-*AGGAAGATGACCCTCCTTCTG* Rw-*GTGAGGAATCTCGGAGGCTG*	302	Exon 3	X69473.1
ADRB3	Fw-*GCACAGAGTAGGGAATCAAAGT* Rw-*CCTTCGGGGCTATCAGACTTTC*	374	Intron	AF109928.2

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unit (Hoefer Scientific SE600, 160×140×1 mm), in a 1x TBE of buffer. The gels were stained with 0.1% silver nitrate and visualized through 2% NaOH solution (containing 0.1% formaldehyde).

DNA Sequencing

Twenty randomly chosen samples of PCR products from homozygote and heterozygote animals were utilized in sequencing. Primers, dNTP, buffer ingredients and nonspecific products were isolated, and then sequencing while using a 3730 sequencer (Applied Biosystems 3730xl DNA Analyzer) was carried out by Bioneer Company in South Korea. The sequenced fragments were then aligned next to each other and SNP identified through sequence traces in contrast of the original sequence in livestock genomics database (http://www.livestockgenomics.csiro.au/blast/). Sequence data generated was further analysed by using laser gene software (Burland, 2000). Multiple sequence alignment was performed using MegAlign programme of Lasergene software.

Statistical Analysis

For *IGF-I* and *ADRB3* genes, allele and genotype frequencies for the Hardy-Weinberg equilibrium test (using χ^2 test) were estimated utilizing GENALEX 6.4 software (Peakall and Smouse, 2006). In order to correct the phenotypic records for fixed effects, Least Square Analyses were conducted through GLM procedure of the SAS software package (SAS 2009) fitting the following linear model.

$$Y_{ijklmn} = \mu + F_i + S_i + YB_k + BT_l + AgeD_{ijklm} + BM_{ijklmn} + b(age - age)_{ijklmn} + e_{ijklmn}$$

Where, y_{ijkl} was the dependent variable, μ the overall mean, F_i was the fixed effect of flock (1 and 2); S_j the fixed effect of sex (male and female), YB_k the fixed effect of year of lambing (1966 to 2003), BT_L the fixed effect of birth type (single, twin or triplet), $AgeD_{ijklm}$ the fixed effect of dam's age, BM_{ijklm} the fixed effect of birth month (January, February, March), b the regression coefficient of lamb age on body weight and finally e_{ijklm} the random residual effect. The 2-way interactions between the fixed effects in the model were tested, but none were significant. Following a correction of the phenotype records for significant fixed effects, association between genotypes and the corrected phenotypes was established as follows:

$$\text{Ccorrected phenotype}_i = G1_i + G2_j + e_{ijk}$$

Where, *G1* and *G2* were the fixed effects associated with the genotypes in *ADRB3* and *IGF-I* genes. The data were presented as least square means±SEM (Standard Error of Mean) with a *P*-value of ≤ 0.05 being considered as statistically significant.

RESULTS AND DISCUSSION

Following an optimization of the parameters that affect the detection of SSCP, the PCR products from 190 animals were analyzed, with an application of the conditions described in the Materials and Methods section. Evaluation of SSCP results revealed two banding patterns for each of *IGF-I* and *ADRB3*. SSCP analysis of the ovine *ADRB3* gene is shown in Figure 1-a. Representative sheep for the two unique SSCP patterns corresponding to two allelic variants A and B are also presented.

SSCP analysis of a 302-bp fragment from Exon 3 *IGF-I* is showed in **Error! Reference source not found.**1-b. Similar to *ADRB3*, two genotypes of AA and AB were observed.

Allele and Genotype Frequencies

The distribution of the frequencies of alleles and the observed genotype frequencies at the IGF-I and ADRB3 loci are presented in Table 2. Only two banding patterns were observed in Baluchi sheep

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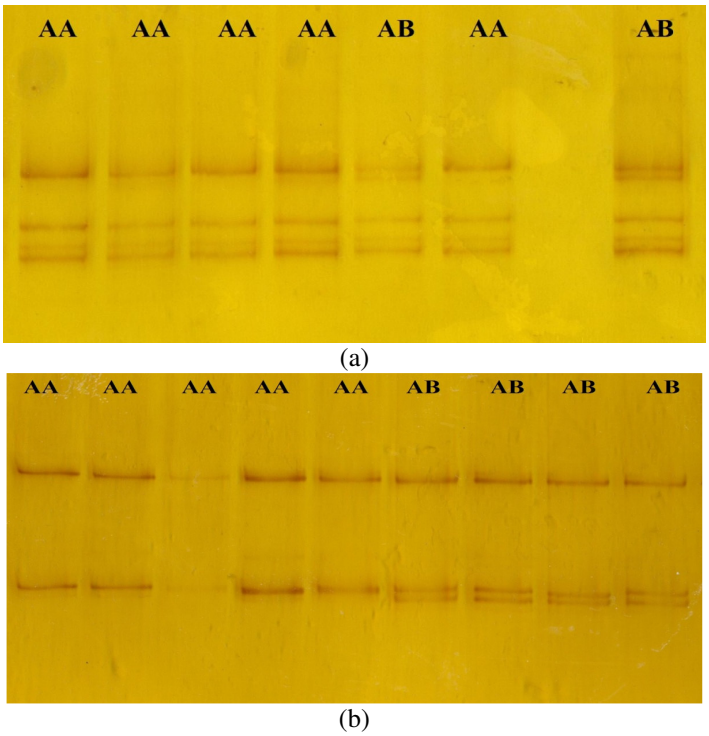

Figure 1. PCR-single strand conformational polymorphism (SSCP) analysis of (a) a 374-bp fragment from *ADRB3* gene (b) of a 302-bp fragment from Exon 3 *IGF-I* gene, in the Baluchi sheep.

population for every gene. In *ADRB3* locus, the frequency of wild type allele (A, 0.93) was higher than that of the mutant allele (B, 0.07) with the genotype frequencies of AA, AB and BB being 0.85, 0.15 and 0.00, respectively. The values from the Chi-square ($\chi 2$) test for genotype frequencies indicated that all the studied loci stand in Hardy-Weinberg equilibrium.

The results obtained from the present study show that the investigated mutations of *ADRB3* that are of a major effect on the regulation of energy metabolism are existent in Baluchi sheep. These results are in agreement with reports in Merino of New Zealand (Forrest *et al.,* 2007; Yang *et al.,* 2009). The authors found six alleles (A-F) but in the present study, only two conformational patterns (genotype) for the *ADRB3* gene were observed.

A study by Tahmoorespur *et al.* (2009) using PCR-SSCP analysis exon1of *IGF-I* revealed three distinct conformational patterns. The genotype frequencies were 44% for pattern AA, 47% for pattern AB and 9% for pattern BB (Tahmoorespur *et al.,* 2009). Yilmaz *et al.* (2005) found the same patterns corresponding with three genotypes of A/A, A/B, and B/B in a mixed breed sheep. However, in this study on exon3 of *IGF-I*, just two banding patterns (AA and AB) were observed. There may be two

Table 2. Allele and genotype frequencies of *IGF-I* and *ADRB3* loci in Baluchi sheep population.

		Allelic frequencies		Observed genotypic frequency			
Gene	No. of sheep	A	B	AA	AB	BB	*P*-values
IGF-I	190	0.95	0.05	0.89 (170)[a]	0.11(20)	0.00 (0)	0.444^{ns}
ADRB3	190	0.93	0.07	0.85 (162)	0.15 (28)	0.00 (0)	0.273^{ns}

[a] Numbers in parentheses indicate the number of individuals that belong to the respective genotypes.

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reasons, first, Baluchi sheep might only have two genotypes, and ,second, it might be due to the the low sample size in the study (190 animals), where the animals with a genotype of BB were not sampled. Perhaps other researches with more animals would be needed to confirm the findings.

Sequencing Results

The results obtained following SSCP analysis were confirmed by sequencing 20 randomly chosen DNA samples for each primer pair, in both forward and reverse directions and for *IGF-I* and *ADRB3*. throughout the study, DNA sequencing analysis indicated that the sequences of PCR products are in correspondence with the sequences in the GenBank. SSCP analysis and sequencing allowed the detection of a nucleotide variation for *ADRB3* and a single nucleotide change for *IGF-I* (Figure2). Sequence analysis revealed a point mutation at position 1620 of the sequence with accession number AF109928 (C to A transversion). A nucleotide change (C → T) was detected in Baluchi breed at nt 271 of *IGF-I*. The same mutation had been already detected in 8 different sheep breeds selected throughout Europe (Pariset *et al.,* 2006) and in three Italian dairy sheep (Scatà *et al.,* 2010). These researchers found that allele T of SNP g.271C> T exerted a positive effect on maintaining a constant milk yield level during lactation. In this case, it is necessary to carry out further studies to better understand the importance of this mutation, In this research, the B allele was defined as the nucleotide sequence with the C→T mutation, and also the A allele as the sequence without the mutation. In a study by Zhang *et al.* (2008) on detection of polymorphism of 5′-regulatory region and five exons of the *IGF-I* gene in the Nanjiang Huang Goat, except for exon 4, no polymorphism was observed in the other amplified regions.

Yang *et al.* (2009) found ten SNPs in Chinese sheep in *ADRB3* Gene. Although 190 sheep were studied in this research, it is still a much smaller study than the previous works, which have revealed eight alleles and have involved 22,558 New Zealand lambs (Byun *et al.,* 2008; Forrest *et al.,* 2003; Forrest *et al.,* 2007) as well as thirteen alleles in 500 Chinese sheep (Yang *et al.,* 2009). This suggests that if more sheep are investigated, more alleles are probable to be found.

Associations between the Growth Traits and Studied Genes

Least square means±SE of pre- and post-weaning growth traits of Baluchi lambs and their association with the above mutations (Figure2) in *IGF-I* and *ADRB3* genes are given in Table 3. In sheep, polymorphisms

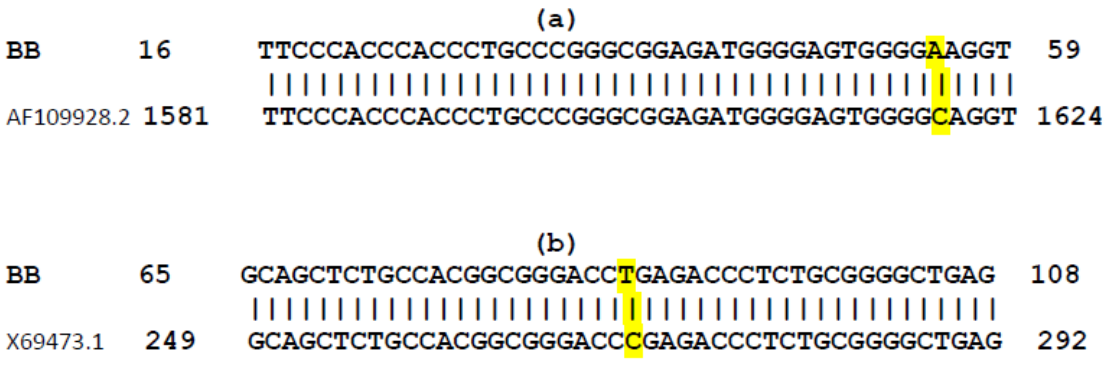

Figure 2. Nucleotide sequence comparison of the PCR products of primers AF109928 and X69473.1: (a) BLAST results of the nt sequence of genotype BB of *ADRB3* with the previously published nt sequence in GenBank, (b) nt sequence of genotype BB of *IGF-I* (the yellow color pointed to the mutation site).

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in the ovine *ADRB3* and *IGF-I* genes have been reported to be associated with growth traits (Forrest *et al.,* 2003; Forrest *et al.,* 2007; Zhang *et al.,* 2008), but in the present study (except body weight in 6MW), there were no significant associations observed between the studied genes and the investigated growth traits. A common assumption in animal breeding is that variation in such quantitative traits as growth or lactation is controlled by many genes. Usually, each one of these genes contributes a limited effect. However, the major gene model suggests that few genes may account for a relatively large proportion of genetic variation. Perhaps these results indicate that growth traits are controlled by a great number of genes, therefore a single gene in its own couldn't capture a remarkable variation of these quantitative traits.

The association of *ADRB3* with 6MW shows that genes' action within various times is different. Human studies have linked variation within the b3-adrenergic receptor gene (ADRB3) to metabolic disease and obesity (NCBI OMIM No. 109691), which suggests that *ADRB3s* present a pivotal role in energy metabolism. In sheep, it has been demonstrated that ADRB3 polymorphism is associated with birth weight, growth rate, carcass composition and lamb survival (Forrest *et al.,* 2003; Forrest *et al.,* 2006; Forrest *et al.,* 2007). The stydy's results are in agreement with Horrell *et al.* (2009) who reported that the ADRB3 gene is an important regulator of pre-weaning growth rate, but affects birth weight only to a limited extent.

CONCLUSIONS

Results within the Baluchi sheep flock revealed that there did not exist any significant associations between *ADRB3* gene and the investigated growth traits (except 6 months weight). This is the first report of the mutation in *ADRB3* gene in Iranian sheep breeds. Through more research works, the variation detected at the *ADRB3* locus may assist in the genetic selection for the desirable animal production traits. The results of the present study indicted that there are two genotypes in exon 3 of *IGF-I* gene in Iranian Baluchi sheep breed, but there wasn't any significant association observed between this gene and growth traits. A previously reported SNP was confirmed in exon 3 of *IGF-I*. It has been reported that this mutation presents a positive effect on maintaining a constant milk yield level during lactation. Therefore, it is necessary to carry out further studies to better understand the importance of this mutation in Iranian Baluchi sheep.

Table 3. Least square Means±SE of pre- and post-weaning growth traits of Baluchi lambs.

Effects		Traits[a]				
		BW	WW	6MW	9MW	YW
IGF-I		ns	ns	ns	ns	ns
	AA	2.30±0.34	21.28±1.18	29.18±1.76	31.87±1.44	39.69±1.65
	AB	2.56±0.41	28.69±1.41	28.69±2.09	31.10±1.72	39.89±1.97
ADRB3		ns	ns	**	ns	ns
	AA	2.42±0.20	20.97±1.25	30.20±1.85	31.34±1.52	39.90±1.74
	AB	2.44±0.22	20.49±1.33	27.67±1.98	31.62±1.62	39.68±1.85

[a] BW= Birth Weight; WW= Weaning Weight; 6MW= 6 Month Weight; 9MW= 9 Month Weight; YW= Yearling Weight.
*: $P<0.05$; **: $P<0.01$, and ns: non-significant (P> 0.05).

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چند شکلی ژنهای *IGF-I* و *ADRB3* و ارتباط آنها با صفات رشد در گوسفندان نژاد بلوچی ایران

ا. قلی بیکی فرد، م. امین افشار، و م. حسین‌پور مشهدی

چکیده

گوسفند بلوچی پر جمعیت‌ترین گوسفند ایران بوده و حدود ۳۰ درصد از جمعیت گوسفندان کشور (تقریباً ۱۵ میلیون راس) را تشکیل می‌دهد. این پژوهش جهت شناسایی جهش‌های موجود در ژنهای IGF-I (اگزون ۳) و ADRB3 (اینترون) و همچنین ارتباط آنها با صفت رشد در گوسفندان بلوچی انجام گرفت. بعد از استخراج DNA از ۱۹۰ گوسفند، برای تکثیر قطعات مورد نظر در دو جایگاه ژنی از دو جفت پرایمر اختصاصی و واکنش زنجیره‌ای پلی‌مراز (PCR) استفاده گردید. برای تعیین الگوهای باندی از روش چند شکلی فضایی تک رشته‌ای (SSCP) و همچنین توالی یابی استفاده گردید. برای ژن IGF-I دو الگوی باندی AA (۰/۸۹) و AB (۰/۱۱) و برای ژن ADRB3 نیز دو الگوی باندی AA (۰/۸۵) و AB (۰/۱۵) شناسایی گردید. توالی یابی (SNP)، در هر کدام از جایگاههای IGF-I و ADRB3 یک تغییر نوکلئوتیدی را نشان داد. تغییر نوکلئوتیدی اگزون ۳ ژن IGF-I قبلا نیز در نژادهای دیگری از گوسفندان دنیا گزارش شده بود. اثرات چند شکلی این ژنها بر صفت وزن (تولد، از شیر گرفتن، شش ماهگی، نه ماهگی و یک سالگی) با روش کمترین مربعات مقایسه شد. بین چند شکلی ژن IGF-I با صفات وزن بدن

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ارتباط معنی‌دار مشاهده نشد، اما ژنوتیپ AA از ژن ADRB3 تفاوت معنی داری با ژنوتیپ ABدر صفت وزن ۶ ماهگی نشان داد($P\leq0.05$).

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