IJABBR- 2014- eISSN: 2322-4827

International Journal of Advanced Biological and Biomedical Research

Journal homepage: www.ijabbr.com

Research Article

Study on DGAT1-exon8 Polymorphism in Iranian Buffalo

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ARTICLE INFO

Article history:
Received: 17 May, 2014
Revised: 08 June, 2014
Accepted: 5 July, 2014
ePublished: 30 July, 2014

Key words:
DGAT1 gene
Iranian buffalo
PCR-SSCP
Milk yeild

ABSTRACT

Objective: Diacylglycerol acyltransferase 1 is a microsomal enzyme that catalyzes the final step of triglyceride synthesis. The objective of this project is to check out the polymorphism at the exon 8 region of DGAT1 gene using PCR-SSCP technique in Iranian water buffaloes (*Bubalus bubalis*). Recent activities have shown that a significant association between lysine at amino acid position 232 with higher milk fat content, whereas an alanine at this position is marker data of lower milk fat content and higher milk yield. **Methods:** In this study we analyzed buffaloes from 5 provinces of Iran. Three PCR-SSCP patterns were found. **Results:** DNA sequencing analysis showed that all of the patterns had the motif "AA" at position 6962-6963 (K allele) encoding lysine, which is similar to "AA" at position 10433-10434 in *Bos indicus* and also different buffalo breeds at position 6962-6963. Therefore exon 8 is a conservative region in *Bos indicus* and all buffalo breeds which is associated with higher milk fat content. Moreover, we found a base transition at position 7036 C/T and we have heterozygosity of both "C and T", which is located at intron 8 of DGAT1 gene in few samples. **Conclusions:** this transition is a nonsense mutation which causes no change in amino acid sequence. This mutation has occurred at the intron 8 of DGAT1 gene in Iranian buffaloes.

INTRODUCTION

Diacylglycerol acyltransferase1 (DGAT1: EC 2.3.1.20) is a key enzyme. In literatures, it is an important enzyme in the triacylglycerol synthesis as a microsomal enzyme. This enzyme acts as catalyzer in the final step of triglyceride synthesis, using diacylglycerol and acyl CoA as substrates (Cases et al., 1998).

In cattle, DGAT1 has a strong functional role and is a positional candidate gene for milk traits, such as milk yield and milk fat content (Furbass et al., 2006; Grisart et al., 2002, 2004; Smith et al., 2000; Spelman et al., 2002; Winter et al., 2002). DGAT1 is located within a region on bovine chromosome 14 that contains a quantitative trait locus for milk production and composition trait (Coppieters et al., 1998; Zhang et al., 1998). Association of two DGAT1 alleles that differed with respect to a lysine-to-alanine substitution K232A at position 10433-10434 in exon 8 with QTL alleles for high and low milk fat percentage has been reported (Grisart et al., 2002; Winter et al., 2002). They stated that this candidate gene is associated with K232A substitution for milk fat percentage in *Bos taurus.* Thus, the role of this position on milk yield and milk composition traits seems to be very significant in *Bos taurus* and it might be true for water buffalo populations as well.

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Buffaloes are known as major milk producers in India, with a milk fat content ranging from 7% to 14% among different breeds (Tantia et al., 2006). DGAT1*K* is reported as a fixed allele in five Indian buffalo breeds that is related to high milk fat content trait (Tantia et al., 2006). Comparison of the Indian water buffalo DGAT1 sequence to the *Bos taurus* indicated 10 synonymous changes in the coding sequence and two non-synonymous changes (Mishra et al., 2007). The first non-conservative changes was a double SNP at nucleotide 10433 and 10434 (AA to GC) resulting in the previous reported change of K232A (A allele) in *Bos taurus* which has been related to lower fat yield and higher milk yield (Spelman et al., 2002; Thaller et al., 2003; Weller et al., 2003). All the Indian and Chinese buffalo breeds as well as *Bos indicus* cattle have a fixed K allele which is responsible for higher milk fat content in buffalo and *Bos indicus* cattle (Kaupe et al., 2004; Lacorte et al., 2006; Tantia et al., 2006; Yuan et al., 2007). Uruguayan Creole cattle (*Bos taurus*) had polymorph DGAT1 allele frequencies (A allele 0.89: K allele 0.11), which are similar to *Bos taurus* beef breeds. The *Bos taurus* breeds selected for milk production and these results came from artificial selection for increasing milk yield (Spelman et al., 2002; Kaupe et al., 2004)

Since buffalo yields higher milk fat and lower milk yield (K allele) than *Bos indicus* or *Bos taurus* cattle, investigation the characteristics of DGAT1 gene in Iranian buffalo was of major interest.

The aim of this study was to examine the polymorphism in exon 8 of the DGAT1 gene in Iranian buffaloes using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) that allows the identification of single nucleotide polymorphisms (SNPs). Buffaloes are of high economic importance to farmers in special provinces of Iran as well as other regions of the world, including in Middle East, India, China and North Africa.

2. MATERIALS AND METHODS

2.1. Sampling and DNA isolation

In this study a total of 50 blood and 190 hair shaft samples were collected from five local Iranian buffaloes from different provinces: Mazandaran (25), Khuzestan (52), Guilan (25), Azerbayjan (119) and Kermanshah (19). Blood samples were collected from the vena jugularis of each buffalo into 4-mL tubes containing EDTA, moreover hair shaft samples were collected from buffaloes labeled and stored at -20°C till DNA extraction. Isolation of DNA was based on the salting-out procedure (Miller *et al*., 1988). The quality and purity of the DNA was assessed by 1% agarose gel electrophoresis and obtained ratio of spectrophotometry (A_{260}/A_{280}) equal to 1.8 that is optimized for a qualified extracted DNA (Figure 1).

2.2. Primer design, PCR amplification and PCR-SSCP method

The primers used for amplification of the buffalo exon 8 of the DGAT1 gene were designed by the Oligo analyzer and Vector NTI and Primer 3plus programs based on the buffalo DGAT1 sequence (GenBank Accession no. DQ886485). Primer sequences were:

Forward5′-CAAGGCTGGTGAGGGCTGC-3′

Reverse 5′-GGGGCGAAGAGGAAGTAGTAG-3′

PCR reaction were performed in a 25 µl volume, using 100 ng of genomic DNA, 2X PCR master Mix containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM of each primer and 0.5 U taq polymerase (Ampliqon, Denmark). The PCR profile included 4 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 61°C and 1 min and 30 sec at 72°C respectively, the final extension is 10 min at 72°C. The length of amplified fragment was 278 bp (Figure 2).

6 microliters of each PCR product was mixed with 12 µl of formamide loading buffer (0.05% xylene-cyanole, 0.05% bromophenol blue, 5.5 mM EDTA, pH 8 in 98% formamide) denatured at 98°C for 12 min and snap-chilled on ice for 15 min. then samples were loaded on a 12% polyacrylamide gel (Acrylamide: Bis-acrylamide ratio of 37.5:1) and electrophoresis has done at 4 °C in 0.5X TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8.3) for 16 h at 250 V. The bands were silver stained (Bassam et al.,1991). 3 PCR products corresponding to different PCR-SSCP patterns purified and sequenced by BIONEER Co. in Korea (Figure 3).

Three PCR-SSCP patterns were observed in Iranian buffaloes. The patterns demonstrated as genotypes AA, AB and BB for the buffaloes from 5 provinces.

Three PCR-SSCP patterns were observed in Iranian buffaloes. The patterns demonstrated as genotypes AA, AB and BB for the buffaloes from 5 provinces.

3. Results and Discussion

The sequences of 278 bases for the Iranian buffalo exon 7 to exon 9 of DGAT1 gene were aligned with the sequence of 8717 bases for the Indian buffalo complete CDS (DQ886485), Murrah buffalo breed partial CDS (FJ014704), Pandharpuri buffalo breed partial CDS (FJ014706), Chinese buffalo complete CDS (AY999090),

Bos indicus partial CDS (DQ228762) and *Bos taurus* complete CDS (AJ318490).

We found that the gene in buffalo coded 489 amino acids which is similar to the sequences above the K232A position, encodes "AA" that leads to K allele or lysine in cattle and buffalo, except *Bos taurus* sequence revealed by Winter et al., 2002. K232A position encodes "GC" and leads in A allele or alanine. The position of K232A in cattle is 10433-10434 (GC/AA) but in buffalo the position is 6962-6963 at exon 8. A substitution that is a transition mutation (C/T) at position 7036, located at intron 8, which is a non-synonymous mutation, was seen in Iranian buffalo which was similar to Indian buffalo (Venkatachalapathy et al., 2013; Winter et al., 2002).
Genotypic and allelic frequencies obtained from PCR-SSCP analysis by GeneAlex6.5 program are demonstrated in table 2.

The BB genotype resembles only T at position 7036 that rarely was seen only in Azerbayjan population. In Indian Murrah buffalo population the frequency of BB genotype was reported 0.09 (Raut et al., 2012).

The SNP which is identified at position 7036, based on accession number: DQ886485, was seen as exchange of C to T. On the other hand both nucleotides (C and T) have been seen in some individuals as heterozygous genotypes, was shown as Y in figure 3, 4 and 5.

It is shown in Figure 4, the first sequence line is the reference sequence from Indian *Bubalus bubalis*, with Accession no. DQ886485 and the other sequence lines belong to Iranian buffalo samples. The C/T substitution as a transition mutation occurred in the population of Iranian buffaloes which is a nonsense mutation.

In figure 5, the heterozygousity for position 7036 is visualized. C and T alleles both are present at this position.

Our finding about the previous SNP (C,T) is similar to the sequencing study on Indian buffalo in Murrah and Pandharpuri breeds at position 7036 (Raut et al., 2012) that was reported at intron 8 region of DGAT1 gene, hence, present and of Raut *et al.*, 2012 studies are consistent, at this position all three genotypes were available in Iranian and Indian Murrah buffalo breeds, as is shown in table 2 with considering the SNP (C,T).

Genotype BB was not seen in Indian Pandharpuri breed (Raut et al., 2012). Based on another study on Indian buffalo and *Bos indicus* cattle breeds a transition mutation C to T at 14th position of intron 8 was reported, and this position was at nucleotide 7036 in *Bubalus bubalis* and 10507 in *Bos indicus* (Venkatachalapathy et al., 2013). In their study only 2 genotypes reported and the genotype BB which represented TT individuals was not reported and table7 denotes genotypic and allelic frequencies related to that study.

The GC/AA substitutions in exon 8 of the DGAT1 gene in the cattle has been associated with milk fat content (Winter et al., 2002; Weller et al., 2003; Grisart et al., 2004). Their achievement agrees with association of DGAT1*K* allele with higher milk fat content, that has been fixed and conserved in *Bos indicus* cattle (DQ228762), Chinese (AY999090) and Indian (DQ886485) buffalo breeds (Tantia et al., 2006; Mishra et al., 2007; Raut et al., 2012). Comparison between *Bos taurus*, *Bos indicus, Bubalus bubalis* and sequences of Iranian buffaloes regarding the sequence of K232A position and the related alignment is shown in figure 6.

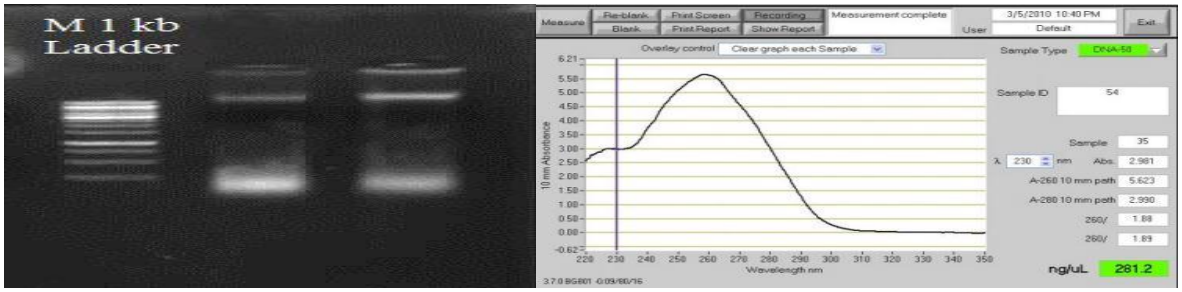

Figure1- 1% agarose gel electrophoresis resembled the quality of DNA and spectrophotometry (A_{260}/A_{280}) electrocardiograph

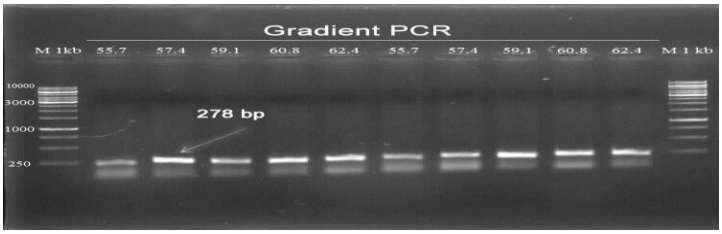

Figure2- PCR Amplification 2% agarose gel electrophoresis

Figure3- Patterns of SSCP derived from 5 provinces in Iran related to Iranian buffalo

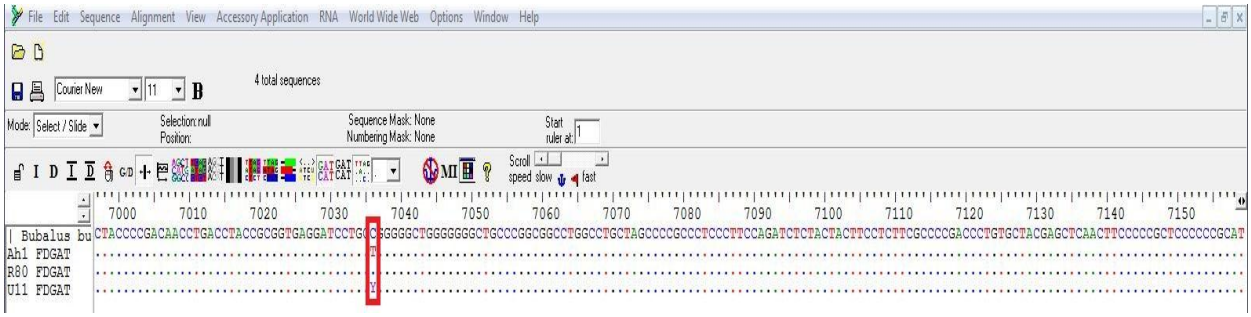

Figure4- C/T substitutions seen in alignments by BioEdit software

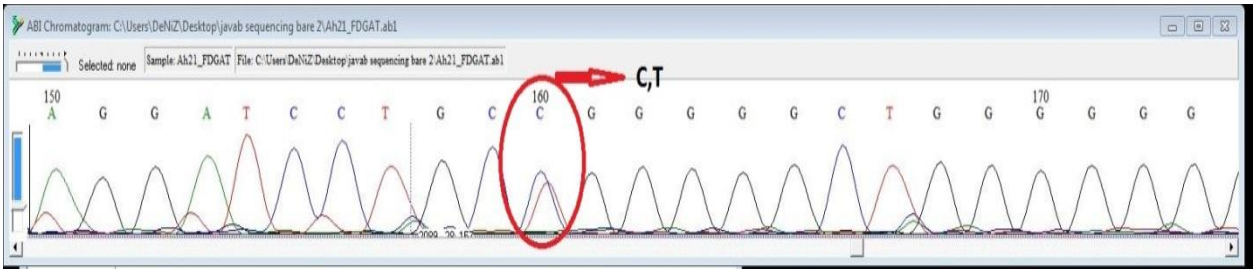

Figure5- The electrocardiograph obtained from sequencing results in BioEdit program represents heterozygous genotypes

Figure6- Alignment of *Bos taurus*, *Bos indicus* (nt10433-10434), *Bubalus bubalis* (nt6962-6963) and the Iranian buffaloes sequences in K232A position

Table 1- Nucleotide substitutions in the PCR-SSCP alleles of the DGAT1 gene in buffalo

Allele	Nucleotide position 7036
A	C
B	T
DQ886485 (*Bubalus bubalis* DGAT1 gene complete CDS)	C

Derived from Accession Number: DQ886485 in GenBank.

Table 2- Genotype and allele frequencies derived from PCR-SSCP analysis of the DGAT1 gene in Iranian buffaloes

Province	No	Genotype frequencies			Allele frequencies	
		AA	AB	BB	A	B
Mazandaran	25	0.56	0.44	-	0.78	0.22
Khuzestan	52	0.38	0.62	-	0.68	0.32
Guilan	25	0.65	0.35	-	0.80	0.20
Azerbayjan	119	0.20	0.78	0.02	0.60	0.40
Kermanshah	19	0.56	0.44	-	0.73	0.27

Table 3-Genotypic and allelic frequencies from Venkatachalapathy *et al*., 2013 report.

	Genotypic frequency		Allelic frequency	
Species and breed	CC	CT	C	T
Buffalo (*Bubalus bubalis*)				
Bhadawari (54)	0.59 (32)	0.41 (22)	0.80	0.20
Mehsana (71)	0.49 (35)	0.51 (36)	0.74	0.26
Murrah (57)	0.54 (31)	0.46 (26)	0.77	0.23
Surti (56)	0.41 (23)	0.49 (33)	0.66	0.34
Overall	0.51 (121)	0.49 (117)	0.74	0.26
Cattle (*Bos indicus*)				
Hariana (40)	0.73 (29)	0.27 (11)	0.86	0.14
Sahival (40)	0.75 (30)	0.25 (10)	0.87	0.13
Nimari (40)	0.82 (32)	0.18 (08)	0.91	0.09
Overall (120)	0.76 (91)	0.24 (29)	0.88	0.12

Conclusions

According to previous studies on *Bos taurus* breeds, AA/GC substitution at position 10433-10434 leads to set alanine amino acid in K232A which is associated with higher milk yield and lower milk fat content (Winter et al., 2002; Weller et al., 2003; Grisart et al., 2004). On the other hand our finding for Iranian buffaloes as well as Indian's reports show GC/AA substitution in exon 8 of DGAT1 at position 6962-6963 in *Bubalus bubalis* that leads to K allele or lysine amino acid to be set in K232A site which seems to be associated with high milk fat content in Iranian buffalo, *Bos indicus*, Chinese and Indian buffaloes. In summary, exon 8 is a conserved region for high milk fat content (K allele) in buffaloes and *Bos indicus* which is not the case in *Bos taurus* breed, based on different studies (Ripoli et al., 2006; Yuan et al.,2007; Tantia et al.,2006; Mishra et al.,2007; Raut et al., 2012).

The SNP which is found at position 7036 of *Bubalus bubalis* in present study and in Raut's report, was at intron 8 region and this substitution might have an important impact on functionality of enzymes such as endothelial nitric oxide synthase (Buraczynska et al.,2004; Gruszcynka et al.,2005). The detected SNP at nucleotide 14th of intron 8 was located in non-coding region, without any direct effect on peptide sequence. It might affect the gene expression and influencing the rate of mRNA splicing (Hastings and Krainer, 2001). For further studies it could be proposed to work on the effects of these SNPs and their relationship and association to biochemical pathways and production traits to reveal their importance in different populations buffaloes.

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