

Novel SNPs of the *ABCG2* Gene and Their Associations with Milk Production Traits in Iranian Holstein Bulls

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ABSTRACT

ABCG2 (ATP binding cassette subfamily G member 2) gene, located on chromosome 6 encodes the *ABCG2* protein that transports various xenobiotics, cytostatic drugs across the plasma membrane as well as cholesterol into milk. A single nucleotide change (A/C) in base 86 of exon 14 is capable of encoding a substitution of tyrosine with serine in the *ABCG2* gene and increase milk yield while decreasing milk fat and protein concentrations. The major aim followed in this research was to study Single Nucleotide Polymorphisms (SNPs) of *ABCG2* gene and their association with milk production traits in Iranian Holstein bulls. Genomic DNA of 105 ified bulls was extracted from semen samples using highly Pure PCR template preparation kit. Primers were designed through Oligo software (Version 5.0) and utilized in PCR. Then the PCR fragments were sequenced. The A/C substitution in base number 86 of exon 14 was observed with 2% frequency which affected protein percentage ($P < 0.05$). Some SNPs were detected for the first time in intron 13, exon and intron 14 in comparison with sequences in the NCBI database. A deletion mutation in base number 20 (T/-) and a missense mutation in base number 67 (A/G) of exon 14 that cause the substitution of serine with glycine were discovered which were significantly associated with protein yield and fat percentage, respectively ($P < 0.05$). Furthermore, significant association was observed between fat percentage and mutations in base numbers 4,133 (T/C) and 4,137 (T/G) of intron 13 ($P < 0.05$). Substitutions in base numbers 2 (T/C) and 55 (G/C) of intron 14 resulted in a significant effect on fat yield and fat percentage ($P < 0.05$).

Keywords: *ABCG2* gene, Holstein bulls, Milk production traits, Polymorphism.

INTRODUCTION

Various studies have provided candidate genes as based on their physiological roles in different traits and diseases. Research of Quantitative Trait Loci (QTL) on chromosome 6 (BTA6) proposed such genes as *PPARGC1A*, *PKD2*, *SPP1*, *OPN* and *ABCG2* as candidate genes which affect milk components (Cohen *et al.*, 2004; Olsen

et al., 2005; Weikard *et al.*, 2005; Olsen *et al.*, 2007).

Many QTL studies demonstrated the effects of the *PPARGC1A* gene on milk production also because of the participation of this gene in fat metabolism (Weikard *et al.*, 2005). Khatib *et al.* (2007) studied associations between transverse mutation A/C at position 3,359 of *PPARGC1A* gene and milk production traits in two Holstein cattle populations. They concluded that A

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allele was associated with significant positive effects on protein percentage and as well a decrease in milk yield in both populations. Olsen *et al.* (2005) proposed *PKD2* gene (polycystin 2) as a candidate gene in dairy cattle. Since calcium is the major osmotic constituent in milk, variations in *PKD2* expression could affect the water content of the milk, and circuitously increase milk fat and protein percentages (Olsen *et al.*, 2007). He suggested three genes (*ABCG2*, *OPN* and *PKD2*) as candidate genes for the QTL.

Cohen *et al.* (2004) suggested that *SPP1* plays a crucial role in mammary gland differentiation and branching of the mammary epithelial ductal system. The *ABCG2* and *SPP1* were expressed in the mammary gland of bovine and increased from parturition through lactation (Cohen-Zinder *et al.*, 2005). Results of research have revealed significant associations between *OPN* gene polymorphism and milk quantitative traits (Leonard *et al.*, 2005; Schnabel *et al.*, 2005; Khatib *et al.*, 2007; Olsen *et al.*, 2007).

ABCG2 gene is a member of ATP-binding cassette transporters which are revealed to deliver xenobiotics and metabolites across the canalicula space into the bile (Leslie *et al.*, 2005). Jonker *et al.* (2005) reported that *ABCG2* was not expressed in virgin mice but was strongly explicated during late pregnancy and especially through lactation. They demonstrated that *ABCG2* expression is responsible for the active secretion of such clinically and toxicologically important substrates as dietary carcinogen PhIP, anticancer drug Topotecan and antiulcerative Cimetidine into mouse milk. The *ABCG2* gene seems to play a key role in stem cell regulation and also in hypoxic defense mechanisms in human (Zhou *et al.*, 2001; Sarkadi *et al.*, 2004). *ABCG2* expression is significantly enhanced during lactation and is accountable for the secretion of vitamin K3 or cholesterol into milk (Van Herwaarden *et al.*, 2007; Farke *et al.*, 2008).

Several SNPs have been identified in the *ABCG2* gene which are of the most

significant associations with milk yield. Fat and protein concentrations were demonstrated for A to C substitution in base number 86 of exon 14, causing a change of the amino acid from tyrosine to serine, at location 581 (Y581S). The mutation was associated with an increased milk yield as well as decreased fat and protein percentages on Bos Taurus (Olsen *et al.*, 2005; Cohen-Zinder *et al.*, 2005; Olsen *et al.*, 2007).

Holstein registered heifers from Europe, the United States, and Canada during were imported 1970s and early 80s to establish the intensive dairy cattle husbandry in Iran. More than 90% of milk presently sold on the free market is Holstein cows' milk. Registered Holstein dairy cow population amounts to about 1 million representing 12.5% of the total cattle population nationwide.

The ultimate aim followed in this study was to detect Single Nucleotide Polymorphism in the *ABCG2* gene as a candidate gene in Iranian Holstein registered bulls and to detect the influence of polymorphisms on milk production traits.

MATERIALS AND METHODS

DNA Extraction and SNP Genotyping

Semen samples were obtained from 105 Iranian Holstein bulls representing resource populations. Genomic DNA was extracted using high Pure PCR template preparation kit (Roche Company kit, CAD No= 11796828001). The quantity and quality of the extracted DNA were assessed through spectrophotometry and electrophoresis on 2 percent Agarose gel. To amplify 240 bp region including partial sequences of intron 13 (base number 4,060 to 4,141), the entire of exon 14 and partial sequences of intron 14 (base number 1 to 68) of *ABCG2* gene, primers were designed through Oligo software (version 5.0). Forward and reverse primers were detected as 5'-GTATTCACGAGACTGTCAGGG-3' and 5'-

GGCTTTATTCTGGCTGTTTCC-3'

respectively. The PCR amplification was adjusted in the best possible condition, 5 μl (150 ng μl^{-1}) of DNA samples being added to 20 μl of PCR mixtures containing 5 μl PCR buffer (10X), 0.5 μl MgCl₂ (1.5 mM), 1 μl of each dNTPs (10 mM), 1 μl of each primer (10 Pmol μl^{-1}) and 0.5 μl of Taq DNA polymerase for (5 unit μl^{-1}). Amplification reactions were conducted in a thermal cycler with an initial denaturation at 95°C for 15 minutes and 35 cycles at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 40 seconds followed by a final extension step at 72°C for 5 minutes.

PCR fragments were purified by means of Qiaquick PCR purification kit (Qiagen Company kit, CAD No= 28104) and then sequenced through ABI 3730 XL 16 Capillary Sequencer and ABI 3730 XL 96 Capillary Sequencer applying Sanger and Dideoxy Chain Termination methods respectively. Eventually, results of sequencing were aligned, making use of Blast software (NCBI) to find similarities vs. differences with the submitted sequence of the *ABCG2* gene in the NCBI database.

Statistical Analysis

Breeding values of quantitative traits were estimated through animal model. Phenotypic records were assessed at first lactation. The records of 305 days and twice milking per day were applied. Only records of cows with calving between 18 and 38 months of age and exceeding 90 days of record were included while animals of no records being excluded. Analyzed traits were Milk Yield (MY), milk Fat Yield (FY), Fat Percentage (FP), milk Protein Yield (PY) and Protein Percentage (PP). Restricted Maximum likelihood Method (REML) based on average information algorithm using ASRMEL programs (version 3.1) was employed to estimate the variance of components (model number 1). The data file included 105 Iran-born Holstein bulls, born between 1983 and 2002. The EBVs were

based on phenotypic records of daughters including 240,000 to 450,000 data items, varying from 59 to 3,632 per bull scattered within 10 to 1,074 herds, recorded from 1967 to 2008.

Model 1) $Y_{ij} = HYS_j + Age_i + A_i + e_{ij}$

Where, Y_{ij} is observation related to favorite traits of i^{th} animal, HYS_j the j^{th} herd-year season combinations effect (as fixed effect), Age_i age at first calving (as a covariate) for i^{th} animal, A_i representing the additive genetic effects of i^{th} animal and e_{ij} standing for random residual effect.

Generalized Linear Model (GLM) was made use of to examine associations between milk quantitative traits and the detected *ABCG2* SNPs using model number 2.

Model 2) $Y_{ij} = \mu + SNP_i + e_{ij}$

In which Y_{ij} is the predicted breeding value for milk production traits related to the i^{th} bull and j^{th} SNP. SNP_i is the i^{th} SNP and e_{ij} the residual random effect. The allele substitution effects were evaluated by regressing EBVs on the number of copies of each allele carried by each animal with the results being presented as the mean \pm standard deviations.

RESULTS

The length of PCR products was equal to 240 bp. Results of sequencing indicated new SNPs in comparison with the recorded sequence of the *ABCG2* gene in the NCBI database (Accession number: AJ871176). All the new polymorphisms were submitted in the NCBI and got the accession number. Statistical results demonstrated significant associations between new mutations in intron 13, exon plus intron 14 and the breeding value of milk quantitative traits.

In intron 13, the T/C mutation (base number 4133, GeneBank accession number: JQ398809) with 7 percent frequency and T/G mutation (base number 4137, GeneBank accession number: JQ398800) with 4 percent frequency showed their significant effects on fat percentage ($P < 0.05$).



A deletion mutation in base number 20 (T/-, Gene Bank accession number: JN811066) with a frequency of 4 percent along with a missense mutation in base number 67 (A/G, Gene Bank accession number: HQ730358) of 8 percent frequency causing the substitution of serine to glycine in exon 14 exerted a significant effect on protein yield and fat percentage, respectively ($P < 0.05$).

A/C mutation (GeneBank accession number: JQ398810) in base number 86 of exon 14 (Y581S) was observed in the Holstein bull population of Iran. Frequencies of allele A and C were recorded as 0.98 and 0.02, respectively. The non-conservative Y581S mutation in *ABCG2* affected protein percentage ($P < 0.05$).

In addition, the results revealed that polymorphisms in base numbers 2 (T/C, GeneBank accession number: JQ398798), and 55 (G/C, GeneBank accession number: JQ398814) in intron 14 with frequencies 13 and 7 percent, respectively had significant influence over fat yield and fat percentage ($P < 0.05$).

The average breeding values of milk quantitative traits were evaluated for wild and mutant alleles of *ABCG2* gene. Results

revealed that, the average breeding values of protein percentage, fat yield and fat percentage were higher for *ABCG2*^A as compared with *ABCG2*^C (base 86 of exon 14), while being lower in milk and protein yields (Table 1).

DISCUSSION

A main goal of dairy cattle genomic research is to identify genes underlying the variation of milk production traits that can be functional in breeding programs. The candidate gene approach, is to supply tools for studying causative SNPs that influence milk components. Genes with a major effect on milk quantitative traits in dairy cattle could be involved in various physiological pathways including triglyceride synthesis diacylglycerol acyltransferase 1 (DGAT1) and ATP binding cassette subfamily G member 2 transporters (*ABCG2*).

The SNP showing significant association with milk components would afford a main opportunity for Marker-Assisted Selection (MAS) programs in livestock (Khatib *et al.*, 2007). A study of the substitutions in *ABCG2* gene alleles and their effects on

Table 1. Estimated average breeding value of milk quantitative traits for SNPs in the *ABCG2* gene (\pm SE).

Gene Region	Allele	Average breeding value of milk traits				
		MY ^a	FY ^b	FP ^c	PY ^d	PP ^e
Exon 14	<i>ABCG2</i> ^A	-160.1(\pm 50.3)	-1.9(\pm 1.32)	0.002(\pm 0.02)	1.9(\pm 1.75)	-0.009(\pm 0.01)
Base 86	<i>ABCG2</i> ^C	294.5 (\pm 287.56)	-2.3(\pm 3.4)	-0.2(\pm 0.06)	4.4(\pm 5.57)	-0.1(\pm 0.03)
Intron 13	<i>ABCG2</i> ^T	-165(\pm 122.32)	-1.68(\pm 6.26)	0.008(\pm 0.07)	1.08(\pm 4.27)	-0.009(\pm 0.03)
Base 4133	<i>ABCG2</i> ^C	-33.7(\pm 52.71)	-5.58(\pm 1.32)	-0.13(\pm 0.02)	9.45(\pm 1.81)	-0.04(\pm 0.01)
Intron 13	<i>ABCG2</i> ^T	-162.2(\pm 219.39)	-1.77(\pm 11.55)	0.006(\pm 0.11)	1.44(\pm 10.54)	-0.01(\pm 0.04)
Base 4137	<i>ABCG2</i> ^G	-5.8(\pm 5.09)	-6.38(\pm 1.2)	-0.17(\pm 0.01)	7.24(\pm 1.75)	-0.03(\pm 0.01)
Exon 14	<i>ABCG2</i> ^T	-160.6 (\pm 239.92)	-1.86(\pm 2.77)	0.0008(\pm 0.07)	1.31(\pm 2.28)	-0.01(\pm 0.04)
Base 20	<i>ABCG2</i> ^T	-45.6(\pm 51.14)	-4.04(\pm 1.34)	-0.05(\pm 0.02)	10.52(\pm 1.78)	-0.01(\pm 0.01)
Exon 14	<i>ABCG2</i> ^A	-163.2(\pm 108.3)	-1.63(\pm 5.99)	0.008(\pm 0.063)	0.88(\pm 3.39)	-0.01(\pm 0.02)
Base 67	<i>ABCG2</i> ^G	-71.6(\pm 53.28)	-5.72(\pm 1.32)	-0.11(\pm 0.02)	11.04(\pm 1.82)	-0.02(\pm 0.01)
Intron 14	<i>ABCG2</i> ^T	-183.6(\pm 192.42)	-1.93(\pm 4.69)	0.01(\pm 0.04)	0.42(\pm 3.99)	-0.009 (\pm 0.02)
Base 2	<i>ABCG2</i> ^C	37.2(\pm 53.53)	-2.02(\pm 1.33)	-0.11(\pm 0.02)	10.43(\pm 1.85)	-0.03(\pm 0.01)
Intron 14	<i>ABCG2</i> ^G	-154(\pm 99.36)	-1.3(\pm 5.14)	0.007(\pm 0.08)	1.67(\pm 3.92)	-0.009(\pm 0.03)
Base 55	<i>ABCG2</i> ^C	-193.3(\pm 52.61)	-12.62(\pm 1.32)	-0.14(\pm 0.02)	1.42(\pm 1.82)	-0.05(\pm 0.01)

^a Milk Yield; ^b Milk Fat Yield; ^c Fat Percentage; ^d Milk Protein Yield, ^e Protein Percentage.

milk components are economically desirable for most selection indexes used in dairy cattle breeding programs, Through which rates of genetic gain could be promoted by direct selection on the alleles.

ABCG2 encoded a protein that is a member of the ATP binding cassette super family (ABC). A wide variety of drugs and various xenobiotics are extruded through the protein and across the plasma membrane (Litman *et al.*, 2000). *A/C* substitution in exon 14, is capable of encoding substitution of tyrosine to serine in the *ABCG2* gene and affect milk quantitative traits (Cohen-Zinder *et al.*, 2005; Olsen *et al.*, 2007). Ron *et al.* (2006) reported allele frequency of *A/C* mutation (Y581S) in 35 breeds. They proposed that the allele *A* of the *ABCG2* gene was predominant in all the populations. The recognition of allele *C* (base 86 of exon 14) only in *Bos taurus* breeds may designate that allele *A* of *ABCG2* is the ancestral and the Y581S the substitution that occurred following a separation of *Bos indicus* from *Bos taurus* genealogy over 200,000 years ago (Ron *et al.*, 2006).

Here, the emphasis is mainly upon the *ABCG2* gene polymorphisms as the most functional candidate gene affecting milk traits in Iranian Holstein bulls. An *A/C* mutation in base 86 of exon 14 was observed with 2% frequency, allele *C* had rare frequency in Iranian Holstein bulls similar to the other breeds. Statistical results also revealed the same influence of *A/C* polymorphism on milk quantitative traits match with the results obtained by other researchers (Cohen-Zinder *et al.*, 2005; Olsen *et al.*, 2007; Komisarek and Dorynek, 2009). New SNPs have been identified in the *ABCG2* gene which are associated with milk quantitative traits. Moreover, the results of the present study revealed that SNPs in *ABCG2* are in association with milk protein and protein percentage traits. In the era of a wide variety of SNP genotypes in animals, it is objective to report estimates out of an optimally fitted model. To be able to recognize such a model, the suitable

model must be fit to be used among the theoretical possible models.

According to the results of the present study, utilization of *ABCG2* gene variations as an index in the Gene Assisted Optimization Index is proposed beside use of other genes affecting traits (such as multiple genes) that are the leading cause of improvement in genetic gain within the domain of animal selection. This study would be functional in Holsteins' breeding programs in Iran. More researches are suggested to study the *ABCG2* polymorphisms as a candidate gene along with its relationships with economical traits, due to its chromosomal position as well as its key role in milk productions.

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چندشکلی های جدید ژن ABCG2 و ارتباط آنها با صفات تولیدی شیر در گاوهای نر هلشتاین ایرانی

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چکیده

ژن ABCG2 (ATP binding cassette sub family G member 2) روی کروموزوم شماره ۶ گاو شناسائی شده که با بیان پروتئین ABCG2 درانتقال مواد دارویی از غشای پلاسما و کلسترول به شیر نقش دارد. در اثر جهش در باز شماره ۸۶ اگزون ۱۴، آلل A به آلل C تبدیل شده و در اثر آن اسیدآمینه تیروزین به سرین تغییر می‌یابد که افزایش میزان شیر و کاهش درصد چربی و پروتئین را به همراه دارد. هدف از این تحقیق بررسی چند شکلی های ژن ABCG2 و ارتباط آن با صفات تولیدی شیر در گاوهای نر نژاد هلشتاین ایرانی بود. DNA ژنومی از ۱۰۵ نمونه اسپرم گاوهای نر هلشتاین تأیید شده در مرکز اصلاح نژاد دام کشور با استفاده از کیت PCR استخراج شدند. آغازگرها توسط نرم‌افزار Oligo (مدل ۵) طراحی و برای تکثیر قطعات مورد نظر استفاده گردید. پس از تعیین توالی محصولات PCR، تعدادی (Single Nucleotide Polymorphisms) SNPs برای اولین مرتبه در اینترون ۱۳، اگزون و اینترون ۱۴ در مقایسه با توالی موجود در NCBI شناسایی شدند. جهش حذفی در باز شماره ۲۰ (T/-)، و جهش معنی دار در باز شماره ۶۷ (A/G) اگزون ۱۴ که در اثر آن اسیدآمینه سرین به اسیدآمینه گلیسین تبدیل می‌شود مشاهده شد که به ترتیب با مقدار پروتئین و درصد چربی ارتباط قابل توجهی داشتند ($P < 0.05$). همچنین ارتباط معنی داری بین جهش باز شماره ۴۱۳۳ (T/C) و باز شماره ۴۱۳۷ (T/G) اینترون ۱۳ با درصد چربی بدست آمد ($P < 0.05$). جابه جایی در باز شماره ۲ (T/C) و ۵۵ (G/C) اینترون ۱۴ اثر قابل توجهی بر روی مقدار و درصد چربی داشت ($P < 0.05$).