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RESEARCH ARTICLE

Variation in the Genetic Effects of *ABCG2*, *Growth Hormone* and *Growth Hormone Receptor* Gene Polymorphisms on Milk Production Traits in Egyptian Native, Holstein and Hybrid Cattle Populations

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ABSTRACT

The aim of the present study was to evaluate the effect of polymorphisms in the ATP-binding cassette subfamily G member 2 (*ABCG2*), *growth hormone* (*GH*) and *growth hormone receptor* (*GHR*) genes on 305-day yields and milk traits in native Egyptian cattle (Baladi), Holstein Friesian cattle and hybrid cattle using DNA sequencing and Restriction Fragment Length Polymorphisms (RFLPs). A total of 148 cows were selected from the three animal populations. The *ABCG2-HhaI*-AA genotype was significantly associated with an increased milk lactose percentage and reduced milk yields in Baladi cows. The *GH-MSP1*-heterozygous genotype (GH) showed significant associations with SNF, protein and lactose percentages in the hybrid cows. Fewer TG repeats were found in the 5' untranslated region of *GHR* in Baladi cows, which could be used for marker-assisted selection to protect against the lower milk yields observed in this population. Additionally, *GHR-MSP1*-VL, can be associated with higher SNF, fat, protein and lactose percentages in hybrid cows, as well as lower milk yields in Baladi cows. We concluded that the three cattle populations have their own genetic identities in relation to genetic markers, which can be used for marker-assisted selection to improve milk traits in these three cattle populations.

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INTRODUCTION

Marked associations between milk yield and composition traits and many genes were recorded in several cattle breeds (Cohen-Zinder *et al*., 2005; Komisarek *et al*., 2011; Ibeagha-Awemu *et al.* 2016; Viale *et al*., 2017). Among these genes, *ATP-binding cassette subfamily G member 2* (*ABCG2*), whose expression is significantly increased in the mammary gland during the lactation period (Farke *et al*., 2008). *Growth hormone* gene *(GH*) plays a crucial role in the growth and development of the mammary gland, as well as the initiation and maintenance of milk secretion; the concentration of *GH* in animals controls the calving interval and the milk yield (Wall and McFadden, 2012). *GH* regulate many other genes that control milk production, such as *insulin-like growth factor* (*IGF*) (Ge *et al*., 2003). Furthermore, its action occurs through the activation of the growth hormone receptor (*GHR*), which has been identified as a strong positional and functional candidate gene that influences milk production (Maskur and Arman, 2014; Parihar, 2016). Mutations in the *GHR* gene alter *GH* functions by changing the protein's ability to bind to this receptor on target cells (Maskur and Arman 2014). Several reports documented the association between milk yield and composition in cattle and the polymorphism in *ABCG2* (Mousavizadeh *et al.* 2013; Ghombavani *et al*., 2016), *GH* (Ge *et al*., 2003; Kim *et al*., 2004; Mullen *et al*., 2010; Komisarek *et al*., 2011; Mullen *et al*., 2011), and *GHR* genes (Blott *et al*., 2003; Maj *et al*., 2005).

Different genetic variants are present at bovine milk trait loci, and considerable differences in allele frequencies for these traits were observed among breeds (Cohen-Zinder *et al*., 2005; Komisarek *et al*., 2011; Alima *et al*., 2013; Ibeagha-Awemu *et al.* 2016; Ozdemir *et al*., 2018). Consequently, marker-assisted selection based on SNPs has been used for the selection of milk production traits (Hayes *et al*., 2007).

Holstein Friesian, Egyptian Baladi (native) and hybrid cattle, which were established several decades ago, represent major cattle populations in Egypt. Under the use of different management systems, their the milk yield and composition are variable, allowing the production of a variety of milk products (Mousavizadeh *et al*., 2013). Therefore, the objective of the present study is to examine milk composition traits (SNF, fat, protein, and lactose percentages) in relation to milk yields in Baladi, Holstein Friesian and hybrid cows and determine their potential associations with polymorphisms in the *ABCG2, GH* and *GHR* genes, as indicated by DNA sequencing and RFLPs in the three cattle populations.

MATERIALS AND METHODS

Animal resources and sampling: A total of 148 animals were selected from three animal populations in Egypt from different farms, including 48 Holstein Friesian, 68 hybrid and 32 Baladi cows. Blood and milk samples were collected from these animals; blood for DNA isolation, and milk for composition analysis, including the SNF, fat, protein and lactose percentages. The 305-day milk yield was also calculated for each animal based on farm records. All Animals were handled in accordance with the recommendations of the Committee on the Ethics of Animal Experiments of Kafrelsheikh University, Egypt.

DNA isolation, PCR amplification, and DNA sequencing: Genomic DNA was extracted from whole blood using the G-spin™ Total DNA Extraction Kit (Intron Biotechnology, South Korea) according to the manufacturer's protocol. PCR amplification of *ATP-binding cassette subfamily G member 2* **(***ABCG2*) (Mousavizadeh *et al*., 2013), *growth hormone (GH*) (Zhou *et al*., 2005) and the 5' untranslated region of the *growth hormone receptor (GHR*) gene were done. The primer sequences, annealing temperatures and amplified fragment sizes used during the amplification of each gene are listed in Table 1. The PCR reaction contained 12.5 µl of the PCR master mix (Thermo Scientific, Fermentas), 2 µl of the DNA, 0.5 µl of the forward and reverse primers (10 pmol/µl) and deionized water up to 25 µl. The PCR protocol was 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, annealing at the temperature listed for each primer in Table 1 for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were purified using the MEGA quick-spin total fragment DNA purification kit (Intron Biotechnology). The purified PCR products from selected samples for each gene were sent to the LGC Company (Germany) for forward DNA sequencing. The sequence data were analyzed using Chromas 1.45 Sequence comparisons were performed using the BLASTN program from the National Center for Biotechnology information website (http://www.ncbi.nlm.nih.gov/). The sequences were aligned using CLUSTAL-W version 1.8 (18). The amino acid translation was achieved using MEGA version 6. The genbank accession numbers used in the comparison of the sequences of the studied genes are listed in Table 2.

Restriction fragment length polymorphisms (RFLPs): The amplified PCR fragments were digested with the restriction enzymes: *Rsa1, MSP1, Hha1, Taq1,* and *HaeIII* (Promega) in a total volume of 25 µl, which was prepared according to the manufacturer's protocol. *Hha1* produced a polymorphic band with *ABCG2*, while *MSP1* produced polymorphic bands in both *GH* and *GHR*. The cleaved fragments were detected via 3% agarose gel electrophoresis. Genotypes were generated for each gene based on the observed restriction enzyme cuts (Table 1).

Statistical Analysis: The phenotypic parameters, means and standard errors were calculated using the PROC MEANS procedure of the SPSS 22 software. Genotypic and allelic frequencies were calculated using the PopGene 32 software. The chi-square test ($\chi 2$) was used to determine whether the populations were in Hardy-Weinberg equilibrium. The association of the identified SNP marker and the studied traits were investigated using the GLM procedures of SPSS version 22.

RESULTS

***ABCG2 gene* sequencing, genotyping and its association with milk composition traits:** 100% sequence similarity was reported for the Holstein cows *in ABCG2* gene sequence compared with *B. Taurus*. Two novels repeated SNPs were observed in both Baladi and hybrid cows: 62437C>T and 62445C>G (Table 2). The latter SNP was located at the recognition site of the *Hha1* enzyme, resulting in two genotypes (AA and AB), their genotype and allele frequencies are listed in Table 3. However, only one genotype (AA) was present in Holstein cattle. Additionally, two different genotypes were found in the examined cattle populations and were not significantly associated with milk composition traits, except for lactose percentage in the case of the Baladi cattle ($P<0.1$) (Table 4).

***GH gene* sequencing, genotyping and its association with milk composition traits:** The comparisons of *GH* sequences with *B. taurus* indicated the presence of three repeated SNPs in both hybrid and Holstein Friesian cows as shown in Table 2. Additionally, Baladi cows showed 100% similarity. *MspI* produced polymorphic genotypes for the *GH* genes in Baladi, hybrid, and Holstein Friesian cattle. The HH, GG and HG genotypes and the H and G alleles are listed in Table 3. In hybrid cattle, heterozygous genotypes (*GH*) showed a significant association with SNF, protein, and lactose percentages ($P<0.1$) (Table 5). Accordingly, the GG genotypes had lower percentages of SNF, lactose and protein than the GH and HH genotypes. Further, a non-significant association was reported for 305-day milk yields and fat% ($P>0.1$). However, this association was not observed in Holstein Friesian cows, in either homozygous or heterozygous genotypes.

Table 1: Primer sequences, their annealing temperature, PCR product size, restriction enzymes used and their generated genotypes

Gene	Primer sequence Forward (5'-3') & Reverse (5'-3')	Annealing temperature (°C)	PCR product size	Restriction enzyme	Digestion product size (bp)
ABCG2	5'- GTATTCACGAGACTGTCAGGG-3 5'- GGCTTTATTCTGGCTGTTTCC-3'	60	240	*Hha1*	(A / A) 240 (A / B) 240, 292, 128 (B / B) 292, 128
GH	5'-CCCACGGGCAAGAATGAGGC-3' 5'-TGAGGAACTGCAGGGGCCC	65	329	*MSP1*	(G / G) 329 (G / H) 329, 224, 105 (H / H) 224, 105
GHR	5'-CTGGCGTATGGTCTTTGTCA-3' 5'-TGGTCTTGCTGCTTTCCTAT-3'	55	318	*MSP1*	(V / V) 318 (V / L) 318, 292, 26 (L / L) 292, 26

Table 2: SNP and different genotypes of *ABCG2*, *GH* and *GHR* genes in Baladi, Holstein and Hybrid cows

	ABCG2 gene		
	Baladi	Hybrid	Holstein
ABCG2	62437C>T	62437C>T	-
Compared with Acc. no AJ871176.1	62445C>G	62445C>G	-
Growth Hormone			
Comparison with gene bank	Compared with *Bos indicus* GH gene, ID: KT001497.1	Compared with *Bos taurus* GH,	Acc. no JQ711182.1
	100% similarity	Ins T 1475	-
		1476 G>C	-
		1575 G>A	1575 G>A
		1576 A>G	1576 A>G
		-	1601 C>T
Comparison with each other	19 C	19 G	19 G
	61 T	61 C	61 C
	62 G	62 T	62 T
	63 G	63 C	63 C
	209 T	209 C	209 T
Growth Hormone Receptor			
No of TG microsatellite in each population	10	14	16
Sequence comparison of the three population	9 SNPS	No SNPs	No SNPs
Compared with gene bank sequences	Compared with *Bos indicus GHR* ID: AF040955.1	Compared with *Bos taurus GHR*, ID: JQ711177.1	
	562 (120833) C>G	120836 G>A	120836 G>A
		120844 G>A	120844 G>A
	TG count 10 vs 10	TG count 14 vs 17	16 vs 17

Table 3: Genotypic and allelic frequencies of assessed *ABCG2*, *GH* and *GHR* genes in Baladi, Holstein and Hybrid cows

Breed	*ABCG2*						*GH*						*GHR*					
	n	AA	AB	BB	A	B	*n*	GG	GH	HH	G	H	*n*	VV	VL	LL	V	L
Baladi cows	32	0.70	0.25	0.0	0.88	0.12	32	0.25	0.00	0.75	0.25	0.75	32	0.62	0.38	0.00	0.81	0.19
Hybrid Cows	68	0.84	0.16	0.0	0.92	0.08	68	0.05	0.16	0.79	0.16	0.84	68	0.32	0.68	0.00	0.66	0.34
Holstein Friesian	48	1.00	0.00	0.0	1.00	0.00	48	0.00	0.27	0.73	0.14	0.86	48	0.73	0.27	0.00	0.86	0.14

n: number.

***GHR gene* sequencing, genotyping and its association with milk composition traits:** A comparison of the sequences of 5´ untranslated region of *GHR* in the three cow populations revealed the presence of 10, 16 and 14 molecular variants of TG repeats in Baladi (*B. indicus)*, Holstein (*B. indicus*) and hybrid cows, respectively.

Nine SNPs were detected in the Baladi cattle in comparison with the Holstein Friesian and hybrid cows. The PCR-RFLP-*MSP1* analysis revealed two genotypes (VV and VL) in the Holstein Friesian, Egyptian Baladi and hybrid cows. The frequencies of these genotypes varied among the three examined cow populations (Table 3).

In Egyptian Baladi cows, *GHR-MSP1* variant genotypes (Table 6) showed a marked association with measured milk composition parameters, including lactose, SNF, fat and protein percentages (P<0.1). The heterozygous (VL) genotype had a higher content of SNF, lactose, fat and protein than the VV genotype. Notably, a non-significant association was found in the case of 305-day milk yields (P>0.1). Additionally, a distinct, strong association with 305-day milk yields was observed in the hybrid cattle population (P<0.05). The VL genotype was associated with a higher milk yield than the VV genotype. However, a non-significant association was recorded for the milk composition parameters (P>0.1). For Holstein cows, the *GHR* genotypes were not markedly associated with either milk yield or milk composition parameters (P>0.1). Moreover, among these different genotypes, the V allele was predominant in the three studied cattle populations.

DISCUSSION

The potential use of genetic polymorphism, particular polymorphism of *ABCG2*, *GH*, and *GHR* genes in some cattle breeds as functional candidates for production traits have been documented in several reports (Lucy *et a*l., 1998; Zhou *et al*., 2005; Hadi *et al*., 2015; Ghombavani *et al*., 2016). In this study we described the use of these markers with three cattle populations, Egyptian native (Baladi), Holstein Friesian and Hybrid cattle, to find out if these markers can be used with these different cattle population for selection and improvement of milk quality and milk yield.

Table 4: Association of different *ABCG2 genotypes* with milk production characteristics in Baladi, Holstein and Hybrid cows

Breed / genotype		AA	AB	P Value
Baladi cows	305 d milk yield (kg)	1804.58±229.83	1830±215.66	0.943
	SNF (%)	8.96±0.128	8.425±0.682	0.100
	Fat (%)	2.83±0.211	2.335±0.724	0.889
	Protein (%)	3.36±0.046	3.15±0.247	0.103
	Lactose (%)	4.97±0.072	4.595±0.378	0.09*
Hybrid cows	305 d milk yield (kg)	2354.22±56.545	2033.33±255.855	0.574
	SNF (%)	9.38±0.060	9.08±0.110	0.604
	Fat (%)	3.94±0.116	3.47±0.821	0.583
	Protein (%)	3.53±0.023	3.41±0.032	0.629
	Lactose (%)	5.09±0.032	4.95±0.068	0.604
Holstein cows	305 d milk yield (kg)	8860±105.59	-	-
	SNF (%)	9.43±0.086	-	-
	Fat (%)	2.67±0.046	-	-
	Protein (%)	3.53±0.032	-	-
	Lactose (%)	5.15±0.047	-	-

Table 5: Association of different *GH* genotypes with milk production characteristics in Baladi, Holstein and Hybrid cows

Breed / genotype		HH	GH	GG	P Value
Baladi cows	305 d milk yield (kg)	1931.938±147.107	-	1448.75±323.1	0.652
	SNF (%)	8.86±0.96	-	9.15±0.88	0.751
	Fat (%)	3.12±0.296	-	3.60±0.156	0.118
	Protein (%)	3.315±0.040	-	3.435±0.040	0.684
	Lactose (%)	4.995±0.061	-	4.95±0.061	0.789
Hybrid cows	305 d milk yield (kg)	2167.679±68.916	2668.75±32.678	2745±67.23	0.675
	SNF (%)	9.447±0.040	9.605±0.061	6.65±0.123	0.006 ***
	Fat (%)	4.204±0.134	3.03±0.050	2.58±0.031	0.131
	Protein (%)	3.556±0.148	3.598±0.023	2.47±0.054	0.005 ***
	Lactose (%)	5.129±0.229	5.238±0.032	3.65±0.002	0.007 ***
Friesian cow	305 d milk yield (kg)	8907.5±104.71	8733.33±429.080	-	0.888
	SNF (%)	9.76±0.123	9.21±0.045	-	0.653
	Fat (%)	2.481±0.085	2.36±0.230	-	0.128
	Protein (%)	3.65±0.017	3.12±0.072	-	0.630
	Lactose (%)	5.325±0.024	5.025±0.099	-	0.213

Table 6: Association of different *GHR* genotypes with milk production characteristics in Baladi, Holstein and Hybrid cows

Breed / Genotype		VV	VL	P Value
Baladi cows	305 d milk yield (kg)	2226.5±71.889	1118.33±58.697	0.223
	SNF (%)	8.77±0.071	9.17±0.338	0.06*
	Fat (%)	2.804±0.420	3.36±0.502	0.05*
	Protein (%)	3.295±0.306	3.433±0.119	0.07*
	Lactose (%)	4.785±0.353	5.13±0.193	0.05*
Hybrid cows	305 d milk yield (kg)	2948±126.492	2005.962±52.470	0.02**
	SNF (%)	9.505±0.077	9.254±0.060	0.481
	Fat (%)	4.03±0.407	3.8±0.109	0.507
	Protein (%)	3.576±0.326	3.476±0.022	0.530
	Lactose (%)	5.163±0.380	5.033±0.326	0.448
Friesian cows	305 d milk yield (kg)	8448.75±225.451	9956.67±406.84	0.202
	SNF%	9.856±0.181	8.726±1.796	0.271
	Fat (%)	2.71±0.184	2.04±1.004	0.414
	Protein (%)	3.68±0.065	3.26±0.683	0.268
	Lactose (%)	5.376±0.502	4.762±0.969	0.273

In this study, the identified SNPs in *ABCG2* gene were few compared to those reported on gene bank. Mousavizadeh *et al*. (2013) demonstrated that non-synonymous nucleotide substitution in exon 14, which is associated with fat and protein percentages in Holstein cattle, was observed at only a 2% frequency. Additionally, Tantia *et al*. (2006) indicated that the presence of fixed alleles of the *ABCG2* gene is responsible for higher milk fat yields and higher fat and protein percentages in Indian cattle (*B. indicus*). Additionally, Ibeagha-Awemu *et al*. (2016) observed low frequency SNPs in genes influencing cow milk traits using high density genome wide genotyping-by-sequencing. The genotypes of the *ABCG2* gene were significantly associated with the milk lactose % ($P<0.1$) in Egyptian Baladi cows (Table 4). Genotype AA possessed a higher lactose percentage than genotype AB. However, a non-significant relationship was observed for the milk yield, as well as the protein, fat and SNF percentages ($P>0.1$). In addition, a non-significant association with milk yield and content parameters was found in hybrid and Holstein Friesian cattle.

These results are consistent with previous findings of Ron *et al.* (2006), they showed that allele A is more frequent than allele B in 23 out of 35 cattle breeds, including both *B. taurus* and *B. indicus* breeds. In addition, the predominance of the A allele is also proposed in the three cattle populations, suggesting that A is an ancestral allele. The significant association reported for lactose percentages in Egyptian Baladi cows indicates that the *ABCG2-HhaI*-AA genotype is associated with

higher lactose and lower milk yields than the AB genotype. This difference may be due to the negative correlation between milk yield (kg) and lactose (Rangel *et al*., 2017). Thus, the *ABCG2-HhaI*-AA genotype could be incorporated into marker-assisted selection for culling of this genotype due to its association with lower milk yields.

GH-MSP1 polymorphism and its association with milk yield and milk production traits in dairy cattle have been documented in many reports (Wall and McFadden, 2012). The present results are consistent with those of a previous study (Zhou *et al*., 2005) that reported an association between *GH-Msp*I^{+} and more milk protein, as well as an association between heterozygous genotypes and higher milk fat content. Additionally, Falaki *et al*. (1996) found an association between the *GH-Msp*I^{-} genotype and a high milk fat content. Consequently, the *GH-MSP1* polymorphism might be a good genetic tool for improving milk composition traits in hybrid cows, especially for SNF, protein and lactose percentages. These findings confirm previous findings in other dairy cattle populations (Blott *et al*., 2003; Maj *et al*., 2005).

The impact of *GH* on milk yield and composition is controlled by *GHR* which modulate its binding capacity to its target cells (Maskur and Arman, 2014). Molecular variants of TG-repeat and SNPs of 5'-untranslated region of *GHR* gene have the potential to serve as candidate genetic marker for selection in milk production traits (Muhaghegh-Dolatabady *et al*., 2013). Hale *et al*. (2000) suggested that shorter TG alleles are common in *Bos indicus* cattle whereas longer 16- to 20-TG-repeat alleles predominate in *Bos taurus* breeds. Although we could not compare the amount of milk in three examined animal population, the great differences in their milk production are not mainly due to variation in environmental factors but may be associated with lower number of TG-*GHR* repeats.

Another important marker in our study was *GHR-MSP1* variant genotypes (VL and VV) in examined cattle populations. These genotypes were markedly associated with the variation in the milk composition contents of fat, protein, SNF and lactose % in Baladi cows. Heterozygous genotypes (VL) had higher SNF %, fat %, protein % and lactose % along with higher milk yield than homozygous genotypes (VV). This association possibly due to the short TG-GHR microsatellite in case of Baladi cows compared to Holstein Friesian and Hybrid cows. Besides, among these different genotypes, the allele H was reported to be predominant in the three studied cattle population. These findings provide confirmation of the previous findings in other dairy cattle population (Blott *et al*., 2003; Maj *et al*., 2005; Li *et al*., 2013). Therefore, *GHR-MSP1* is tightly associated with the different milk composition characteristics in Baladi cattle.

Conclusions: The three cattle populations have their own genetic identities, which can be used to facilitate marker-assisted selection for each cattle population. The *ABCG2-HhaI*-AA genotype can be used for marker-assisted selection against this genotype due to its association with lower milk yields. The *GH-MSP1* polymorphism might be a good genetic tool for the improvement of milk composition traits in hybrid cows. The presence of fewer TG-*GHR* repeats can be used in marker-assisted selection against lower milk yields, which is a common characteristic in *B. indicus* breeds. In contrast, a greater number of TG-*GHR* repeats and higher milk yields are obtained with *B. taurus* breeds (Holstein Friesian).

Authors contribution: AF conceived and designed study. WM executed the experiment. SEl analyzed the data. SEI revised the manuscript. AF and SEl contributed in the intellectual & scholarly write up of the manuscript. All authors approved the final version for submission.

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