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Polymorphisms of the *IGF1* gene and their association with growth traits, serum concentration and expression rate of *IGF1* and *IGF1R* in buffalo*

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Abstract: The insulin-like growth factor 1 (*IGF1*) gene is a member of the group of somatotropin axis genes that play a significant role in cell proliferation and growth of muscles. Here, we searched for polymorphisms in buffalo *IGF1* and found two novel single nucleotide polymorphisms (SNPs), G64A and G280A, in the noncoding sequences of exon 1 and exon 4, respectively. Statistical analysis of different genotypes showed that the individuals with GG genotypes had significantly (P<0.05) higher body weight (BW) and average daily gain (ADG) than those with other genotypes at ages of 3–6 months in G64A SNP and 6–9 months in G280A SNP. The combined genotypes of these two SNPs produced three haplotypes, GG/GG, AG/AG, and AA/AA, which were significantly associated (P<0.0001) with BW and ADG at an age from 3 to 12 months. Buffaloes with the homozygous GG/GG haplotype showed higher growth performance than other buffaloes. The two SNPs were correlated with mRNA levels of *IGF1* and IGF1 receptor (*IGF1R*) in semitendinosus muscle as well as with the serum concentration level of IGF1. Also, buffaloes with GG/GG haplotype showed higher mRNA and serum concentration levels. The data revealed that these two SNPs could be valuable genetic markers for selection of Egyptian buffaloes for better performance in the population.

Key words: Insulin-like growth factor 1 (*IGF1*); *IGF1R*; Single nucleotide polymorphism (SNP); Growth traits; Buffalo
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1 Introduction

Insulin-like growth factor 1 (IGF1) is essential for activation of mitogenic proliferation and myoblast differentiation in skeletal muscles (Werner *et al.*, 1994; Oksbjerg *et al.*, 2004) and therefore it can be used in marker-assisted selection (MAS) for growth in bovines (Chung and Kim, 2005; Andrade *et al.*, 2008; Islam *et al.*, 2009; Mullen *et al.*, 2011). Sahana

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et al. (2010) have identified the quantitative trait loci (QTL) containing the bovine *IGF1* locus on BTA5. This gene consists of six exons (Fotsis *et al.*, 1990; Grosse *et al.*, 1999; Sahana *et al.*, 2010) with alternative splicing in exon 1 and exon 2 that produce two alternative transcripts (class I and class II). These transcripts are differently expressed in various species and tissues; class I is expressed higher than class II in cattle (Wang *et al.*, 2003). *IGF1* has been characterized as a key regulatory gene which controls postnatal tissue growth in animals (Jones and Clemmons, 1995; Gerrard *et al.*, 1998). Moreover, local IGF1 overexpression in skeletal muscles induces their hypertrophy and improves their strength (Shavlakadze *et al.*, 2010).

Previous studies reported two single nucleotide polymorphisms (SNPs): C512T in the promoter region and rs29012855 in intron 3 of bovine *IGF1* which were significantly associated with growth traits (Ge *et al.*, 2001; Islam *et al.*, 2009; de la Reyna *et al.*, 2010; Mullen *et al.*, 2011). Some other SNPs in the intronic regions had no significant association with growth traits (de la Reyna *et al.*, 2010). So far, only a few studies have investigated *IGF1* polymorphisms in buffalo and they found one transition SNP: G/A within an *Eco*130I site of intron 3 (Dierkes *et al.*, 1999; Fatima *et al.*, 2009). However, these previous reports have not investigated the association of this SNP with performance traits and gene expression. This prompts us to conduct this study to look for *IGF1* polymorphisms and to determine their associations with growth traits, relative gene expression of *IGF1* and IGF1 receptor (*IGF1R*) in skeletal muscle, as well as the serum concentration of IGF1 in Egyptian buffalo.

2 Materials and methods

2.1 Animals

Two hundred (95 females and 105 males) healthy Beheiry buffaloes, maintained at Mahlet Mussa station in Kafrelsheikh Governorate, Egypt, were randomly chosen. All body weight (BW) data were obtained from the farm records. These animals were unrelated genetically to each other (i.e. coming from unrelated sires/dams or grand-sires/grand-dams), as revealed by the farm records. This study was reviewed and approved by Kafrelsheikh University Animal Care and Ethics Committee and was carried out at the Molecular Biology Lab, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt.

2.2 Polymerase chain reaction

The Gene JET genomic DNA extraction kit was used to extract genomic DNA from blood following the manufacturer's protocol (Thermo Scientific, #K0721, European Union). Four loci of *IGF1* gene were amplified by polymerase chain reaction (PCR) using specific primers (Table 1) designed based on the bovine *IGF1* sequences submitted to GenBank with accession number DQ851589. The PCR was carried out as described in previous studies (El-Magd *et al.*, 2013; 2014; Abo-Al-Ela *et al.*, 2014), but with annealing temperatures of 55–60 °C for 40 s. PCR products were checked for size and quality on 1% agarose gels.

2.3 SNP identification by sequencing and single-strand conformation polymorphism

Twenty-five purified PCR products for each locus were commercially sequenced by MacroGen

Table 1 Primers used in this study

Locus	Primer (5'→3')	T_a (°C)	Size (bp)	Localization	Experiment
IGF1.1	F: GGGCAAAAAGCATGAGACAGT R: GCTGATTTTTCCCATTGCTTCTGA	60	785	Exon (E) 1	SNP detection
IGF1.2	F: GCCAGCAGCTCACAAGCTGA R: ACCATTTTTGTTGTTCCAGAT	55	390	E2	SNP detection
IGF1.3	F: TTGCACTCCTGGAAGGGGCATA R: TCTTCGCACACTCCCCGGCAGTT	55	362	E3	SNP detection
IGF1.4	F: CCACTCTAAAGCTAGGCCTCTCTC R: GAAGTCTATGAGGGTATGAAT	55	340	E4	SNP detection
IGF1	F: TTGGTGGATGCTCTCCAGTTC R: AGCAGCACTCATCCACGATTC	60	218	E3–E4	Relative expression
IGF1R	F: AAGAACCATGCCTGCAGAAGG R: GGATCTCAGGTTCTGGCCATT	60	105	E11, E12	Relative expression
β-actin	F: CGACAACGGCTCCGGCATGT R: CTCCTCAGGGGCCACACGGA	60	271	Refer to NM_173979	Relative expression

F: forward; R: reverse; T_a: annealing temperature

(Seoul, Korea). Sequence data were manually checked for quality and their identity was analyzed using the BLAST tool of National Center for Biotechnology Information (NCBI). Identification of SNPs and sequence alignments were performed using Geneious 4.8.4 software. Single-strand conformation polymorphism (SSCP) was performed as described in previous studies (El-Magd *et al.*, 2014).

2.4 Transcription factor binding site analysis

The MatInspector software package was used to determine the effects of allele substitution of SNPs in the noncoding regulatory regions of exon 1 and exon 4 of *IGF1* gene on predicted transcription factor binding sites (TFBSs) (Quandt *et al.*, 1995).

2.5 Quantitative real-time PCR analysis for *IGF1* and *IGF1R*

Total RNA was isolated from the semitendinosus muscle biopsy using a Gene JET RNA Purification Kit including DNase I digestion following the manufacturer's protocol (Thermo Scientific, #K0731, USA) and as described in previous studies (El-Magd *et al.*, 2013). The RNA was then reverse-transcribed using RevertAid™ H Minus Reverse Transcriptase which is a genetically modified moloney murine leukemia virus reverse transcriptase (M-MuLV RT; Thermo Scientific, #EP0451, USA). Following reverse transcription, relative expression of the *IGF1* and *IGF1R* genes was determined using StepOnePlus™ real-time PCR system (Applied Biosystem, USA) with 2× Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, #K0221, USA) and gene specific primers designed by Primer 5.0 software (Table 1). The housekeeping gene (*β-actin*) represented the normalizer, which was used to calculate the relative gene expression or fold change in target genes. The following thermal cycling conditions were used: initial denaturation at 95 °C for 10 min, 40–45 cycles of amplification of DNA denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. At the end of the last cycle, temperature was increased from 63 to 95 °C to produce a melting curve. The quantities of the critical threshold (C_T) of the target gene were normalized with quantities of *β-actin* using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2.6 Measurement of IGF1 serum concentration

Serum IGF1 concentrations were determined by radioimmune assay (RIA) with human recombinant IGF1 as a standard similar to a protocol described by Bishop *et al.* (1989). Each sample was assayed in duplicate at two dilutions and in a single assay.

2.7 Statistical analysis

Allele and genotype frequencies, Hardy-Weinberg equilibrium (HWE), gene heterozygosity (*He*), effective allele numbers (N_e), and polymorphism information content (PIC) were calculated as described in previous studies (Abo-Al-Ela *et al.*, 2014; El-Magd *et al.*, 2014). Haplotype reconstruction and frequencies, and linkage disequilibrium (LD) (measured by LD coefficient (D')) were determined by the Haploview program (Barrett *et al.*, 2005). Associations between *IGF1* gene genotypes/haplotypes and growth trait phenotypes were analyzed using a general linear model (GLM) procedure of SAS V9 (SAS Inst. Inc., Cary, NC, USA). All analyses were performed in two steps: first, a full animal model was used; and second, a reduced animal model was used. The full animal model includes fixed effects of SNP genotype, sex, SNP genotype–sex interaction, age at weighting as a covariate with linear and quadratic regression coefficients as well as random residual error. Statistical significance for the fixed effects was determined using *F*-statistics, and covariate was tested individually. Preliminary statistical analyses indicated that these effects did not have a significant influence on trait variability in the analyzed populations ($P>0.05$). Thus, the reduced model is as follows: $Y_{ij}=\mu+G_j+e_{ij}$, where Y_{ij} represents the value of growth traits (BW at different time points (0 (birth), 3, 6, 9, 12, 18, and 24 months of age) and average daily gain (ADG) measured on the *i*th animal), μ is the overall mean for each trait, G_j is the fixed effect of the *j*th *IGF1* genotype (GG, AG, AA in *IGF1.1* and *IGF1.4* loci) and haplotypes (GG/GG, AG/AG, AA/AA), and e_{ij} is the random residual effect of the *j*th *IGF1* genotype and haplotypes on the *i*th animal. The allelic substitution (α) and dominance effects (δ) were estimated using the same but re-parameterized model. For allelic substitution (α) the SNP effect was treated as a covariate, represented by the number of A alleles at both loci, while for dominance effects (δ) genotypes were

indicated as 0, 1, and 0 for GG, AG, and AA, respectively, according to Falconer and Mackay (1996). The results of the multiple comparisons were corrected by the Bonferroni's correction, and the differences were considered significant at $P<0.05$. Data were reported as mean±standard error of mean (SEM).

3 Results

3.1 Analysis of the detected SNPs

Four loci, *IGF1.1–IGF1.4* containing exons 1–4, respectively, were amplified by PCR (Fig. 1a). Analysis of SSCP results revealed presence of three SSCP banding patterns (GG/AG/AA) in *IGF1.1* and *IGF1.4* loci (Fig. 1b), but other loci showed only one pattern (no polymorphisms). The sequences of these loci were submitted to GenBank with accession numbers KC107767 and KC668318, and their analysis showed two novel SNPs: G64A and G280A in the noncoding region of exon 1 and exon 4, respectively (Fig. 1c).

In silico analysis of TFBSs predicted that the G allele of G64A SNP introduced two new TFBSs (Thing1-E47 and Snail) that are cancelled by the A allele. Additionally, the A allele of G280A SNP formed two new TFBSs (Hen-1 and Snail), while the G allele formed AML-1 (RUNX1) TFBS.

The allelic and genotypic frequencies were calculated for *IGF1.1* and *IGF1.4* (Table 2). The A allele and AA genotype had higher frequencies (0.60 and 0.41, respectively) for G64A SNP, whereas the G allele and AG genotype had a higher frequency (0.55 and 0.43, respectively) for G280A SNP. The χ^2 test showed that the distributions of the three genotypes of

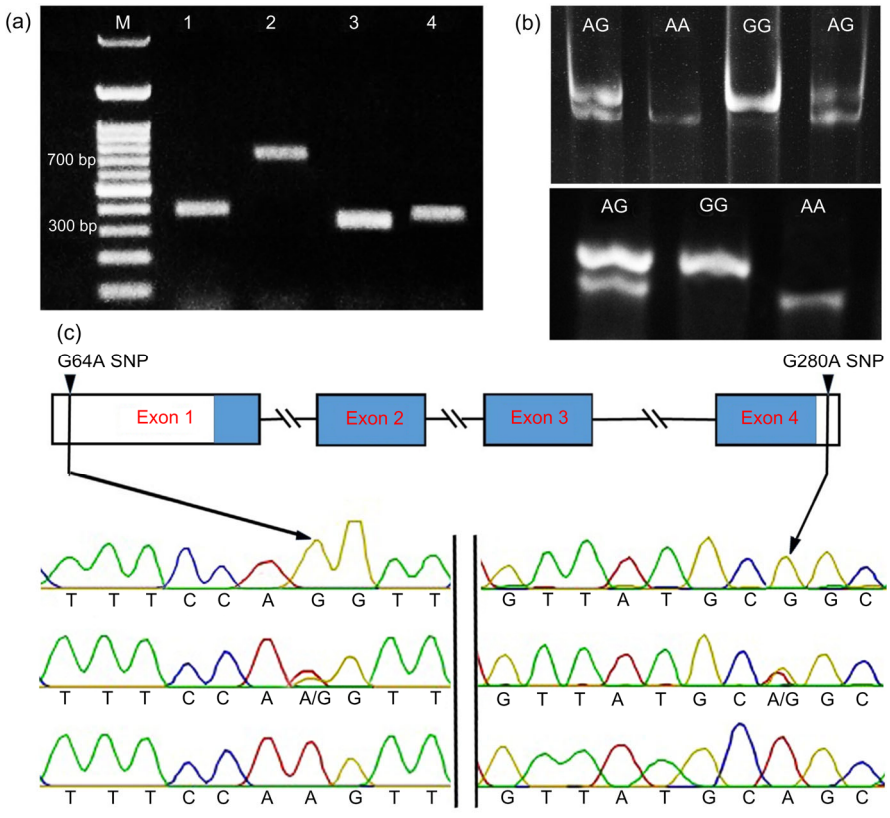

Fig. 1 PCR products, PCR-SSCP patterns, and nucleotide sequences of *IGF1.1–IGF1.4* loci (exons 1–4) in Egyptian buffaloes

(a) Ethidium bromide-stained agarose gel of PCR products: 390 bp exon 2 (Lane 1), 785 bp exon 1 (Lane 2), 340 bp exon 4 (Lane 3), and 362 bp exon 3 (Lane 4); M represents 100 bp ladder. (b) Three SSCP patterns were detected: genotype AG, AA, and GG in *IGF1.1* locus (exon 1) and genotype AG, GG, and AA in *IGF1.4* locus (exon 4). (c) Sequence chromatogram spanning the *IGF1* mutation site shows two single nucleotide polymorphisms (SNPs): G64A and G280A (arrows) located within the noncoding sequence of exon 1 and exon 4, respectively. The blue-colored boxes refer to the coding sequences of exons 1–4 (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

G280A SNP within the Egyptian buffalo population were in Hardy-Weinberg equilibrium (HWE) (P>0.05), whereas three genotypes of G64A SNP deviated from HWE (P<0.05).

The results of the population genetic indexes are presented in Table 2. High heterozygosities were observed in *IGF1.1* and *IGF1.4* loci. Based on the classification of PIC, Egyptian buffaloes possessed medium polymorphism (0.25<PIC<0.5) at *IGF1.1* and *IGF1.4* loci. Pair-wise LD analysis revealed a strong linkage between the two SNPs (D'=1.0) with a minor allele frequency of 0.40 in the *IGF1.1* locus and 0.45 in the *IGF1.4* locus.

A total of 4 haplotypes (GG/GG, AG/AG, GA/GA, and AA/AA) were determined and the haplotypes AA/AA and GG/GG were most common at frequencies of 0.450 and 0.402, respectively, whereas the haplotype AG/AG was a minor haplotype with a frequency of 0.148, and the remaining haplotype (GA/GA) was rare with a frequency of 0.00003 and so was excluded from the association analysis.

3.2 Association of SNP genotypes and haplotypes with growth traits

Associations of individual genotypes and haplotypes at *IGF1.1* and *IGF1.4* loci with the growth traits (BW and ADG) were analyzed in Egyptian buffaloes at 0 (birth), 3, 6, 9, 12, 18, and 24 months of age. The results of this association analysis are shown in Tables 3–5. A significant association was observed between genotypes of the two loci and BW and ADG during the growing stage at ages of 3–6 months in G64A SNP (P<0.05; Table 3) and 6–9 months in G280A SNP (P<0.0001; Table 4). In addition, the three haplotypes AA/AA, AG/AG, and GG/GG were significantly associated with BW and ADG during the growing stage at ages of 3–12 months (P<0.0001; Table 5). In all significant associations, GG genotype and GG/GG haplotype buffaloes have higher BW and ADG performance than those with other genotypes and haplotypes. However, growth trait means did not differ significantly between the AG and AA genotypes or between the AG/AG and AA/AA haplotypes (P≥0.05).

Table 2 Genotypic and allelic frequencies, value of χ^2 test, and diversity parameters and linkage disequilibrium of G64A and G280A SNPs of *IGF1* in buffalo

SNP	Genotype frequency (number)			Allele frequency		χ^2 (HWE)	P-value	*He*	N_e	PIC	D'	MAF
	GG	AG	AA	A	G							
G64A	0.215 (43)	0.375 (75)	0.410 (82)	0.60	0.40	9.94	0.0016	0.48	1.93	0.37	1.0	0.40
G280A	0.335 (67)	0.430 (86)	0.235 (47)	0.45	0.55	3.58	0.058	0.50	1.98	0.37		0.45

χ^2: Chi-square value; HWE: Hardy-Weinberg equilibrium; *He*: gene heterozygosity; N_e: effective allele numbers; PIC: polymorphism information content; MAF: minor allele frequency

Table 3 Association of genotypes at exon 1 of the *IGF1* gene with growth traits in Egyptian buffalo

Time (month)	BW (kg)			Overall P-value	α	δ
	GG	AG	AA			
0	32.14±0.52	33.66±0.64	32.67±0.73	0.325	0.27±0.11	1.26±0.09
3	87.92±1.81^a	80.42±1.32^b	79.91±2.19^b	0.016	−4.01±0.79**	−3.49±0.87*
6	161.92±3.84^a	140.92±4.84^b	143.14±3.91^b	0.007	−9.39±1.04**	−11.61±0.93**
9	189.43±4.12	184.50±4.82	181.26±3.93	0.493	−4.09±1.91	−0.84±0.89
12	227.20±4.52	224.30±5.52	226.30±5.62	0.937	−0.45±0.08	−2.45±0.31
18	290.04±5.72	291.94±6.60	289.28±5.97	0.949	−0.38±0.12	2.28±0.83
24	386.28±12.14	380.69±10.92	374.58±11.85	0.807	−5.85±1.15	0.26±0.09
Time (month)	ADG (kg/d)			Overall P-value	α	δ
	GG	AG	AA			
0–3	0.62±0.01^a	0.52±0.03^b	0.52±0.01^b	0.007	−0.05±0.001*	−0.05±0.02*
3–6	0.82±0.02^a	0.67±0.04^b	0.70±0.02^b	0.005	−0.06±0.01**	−0.09±0.02**
6–9	0.31±0.07	0.48±0.06	0.42±0.05	0.154	0.06±0.03	0.12±0.02
9–12	0.42±0.07	0.44±0.07	0.50±0.06	0.689	0.04±0.01	−0.02±0.01
12–18	0.35±0.03	0.38±0.02	0.35±0.04	0.751	0.00±0.00	0.03±0.02
18–24	0.53±0.07	0.49±0.04	0.47±0.06	0.787	−0.03±0.001	−0.01±0.002

BW: body weight; ADG: average daily gain; α: allelic substitution effect (additive effect); δ: dominance effect. * P<0.05, ** P<0.01 vs. GG genotype. Data are expressed as mean±SEM, with n=43 for GG, n=75 for AG, and n=82 for AA. $^{a, b}$ Means within the same row carrying different superscripts differ significantly at P<0.05 based on Bonferroni's multiple comparison test

Table 4 Association of genotypes at exon 4 of the *IGF1* gene with growth traits in Egyptian buffalo

Time (month)	BW (kg)			Overall *P*-value	α	δ
	GG	AG	AA			
0	31.06±0.75	32.92±0.58	31.81±0.69	0.114	0.38±0.06	1.49±0.41
3	82.79±1.70	80.81±1.15	77.72±1.36	0.073	−2.54±0.37	0.56±0.23
6	156.05±2.51[a]	146.57±2.11[b]	139.80±2.03[b]	<0.0001	−8.13±0.24**	−1.36±0.08**
9	201.43±2.62[a]	180.50±2.21[b]	175.26±2.23[b]	<0.0001	−13.09±0.75**	−7.85±0.91**
12	241.72±9.32	229.30±7.58	230.30±5.63	0.481	−5.71±1.03	−6.71±0.95
18	293.42±9.02	291.54±8.73	282.38±8.87	0.715	−5.52±0.08	3.64±0.14
24	387.35±12.05	384.44±10.62	381.71±11.5	0.951	−2.82±0.38	−0.09±0.01
Time (month)	ADG (kg/d)			Overall *P*-value	α	δ
	GG	AG	AA			
0–3	0.57±0.07	0.53±0.04	0.51±0.06	0.767	−0.03±0.01	−0.01±0.001
3–6	0.81±0.02[a]	0.73±0.01[b]	0.69±0.02[b]	<0.0001	−0.06±0.001**	−0.02±0.001**
6–9	0.50±0.01[a]	0.38±0.02[b]	0.39±0.02[b]	<0.0001	−0.06±0.001**	−0.07±0.001**
9–12	0.45±0.07	0.54±0.06	0.59±0.08	0.377	0.07±0.01	0.02±0.02
12–18	0.29±0.06	0.35±0.02	0.29±0.04	0.488	0.00±0.00	0.06±0.02
18–24	0.52±0.03	0.52±0.02	0.55±0.04	0.678	0.02±0.01	−0.02±0.02

BW: body weight; ADG: average daily gain; α: allelic substitution effect (additive effect); δ: dominance effect. * P<0.05, ** P<0.01 vs. GG genotype. Data are expressed as mean±SEM, with n=67 for GG, n=86 for AG, and n=47 for AA. [a, b] Means within the same row carrying different superscripts differ significantly at P<0.05 based on Bonferroni's multiple comparison test

Table 5 Association of haplotypes at exons 1 and 4 of the *IGF1* gene with growth traits in Egyptian buffalo

Time (month)	BW (kg)			*P*-value
	GG/GG	AG/AG	AA/AA	
0	31.52±0.47	30.45±0.51	31.07±0.26	0.253
3	89.43±1.82[a]	80.81±1.94[b]	78.77±1.22[b]	<0.0001
6	158.82±2.51[a]	139.72±2.38[b]	140.12±2.01[b]	<0.0001
9	207.82±3.81[a]	181.62±3.15[b]	180.25±3.02[b]	<0.0001
12	247.72±5.18[a]	210.37±4.14[b]	212.36±4.03[b]	<0.0001
18	302.24±6.12	292.87±6.90	286.54±6.42	0.188
24	391.30±14.15	388.32±15.11	384.26±15.24	0.939
Time (month)	ADG (kg/d)			*P*-value
	GG/GG	AG/AG	AA/AA	
0–3	0.64±0.02[a]	0.56±0.02[b]	0.53±0.01[b]	<0.0001
3–6	0.77±0.04[a]	0.65±0.02[b]	0.68±0.01[b]	0.011
6–9	0.54±0.02[a]	0.47±0.01[b]	0.45±0.01[b]	<0.0001
9–12	0.44±0.03[a]	0.32±0.02[b]	0.36±0.01[b]	0.001
12–18	0.30±0.09	0.46±0.10	0.41±0.08	0.476
18–24	0.49±0.08	0.53±0.09	0.54±0.09	0.907

BW: body weight; ADG: average daily gain. Data are expressed as mean±SEM, with n=55 for GG/GG, n=80 for AG/AG, and n=65 for AA/AA. [a, b] Means within the same row carrying different superscripts differ significantly at P<0.05 based on Bonferroni's multiple comparison test

3.3 Association of SNP haplotypes with expression of *IGF1* and *IGF1R* and serum concentration of IGF1

The increase in growth traits may be accompanied by change in the expression levels of *IGF1* and *IGF1R*. To check this possibility, relative quantitative PCR (qPCR) was used to estimate the expression levels of these genes in semitendinosus muscle of high GG/GG and low AG/AG and AA/AA growth performance buffaloes at ages of 3, 6, 9, and 12 months. Interestingly, the expression levels of the two genes were significantly (P<0.05) higher in GG/GG buffaloes than in other buffaloes, whereas no significant (P≥0.05) differences in gene expression were detected between buffaloes with AG/AG and AA/AA haplotypes (Fig. 2). Similarly, there was a significant (P< 0.05) change in the serum concentration of IGF1, with GG/GG haplotype buffaloes having a higher serum concentration of IGF1 than those with other haplotypes (Fig. 3). Collectively, GG/GG haplotype buffaloes had superior growth traits, higher expression levels of *IGF1* and *IGF1R* mRNA, as well as higher serum concentration of IGF1 than AG/AG and AA/AA buffaloes at 3–12 months of age.

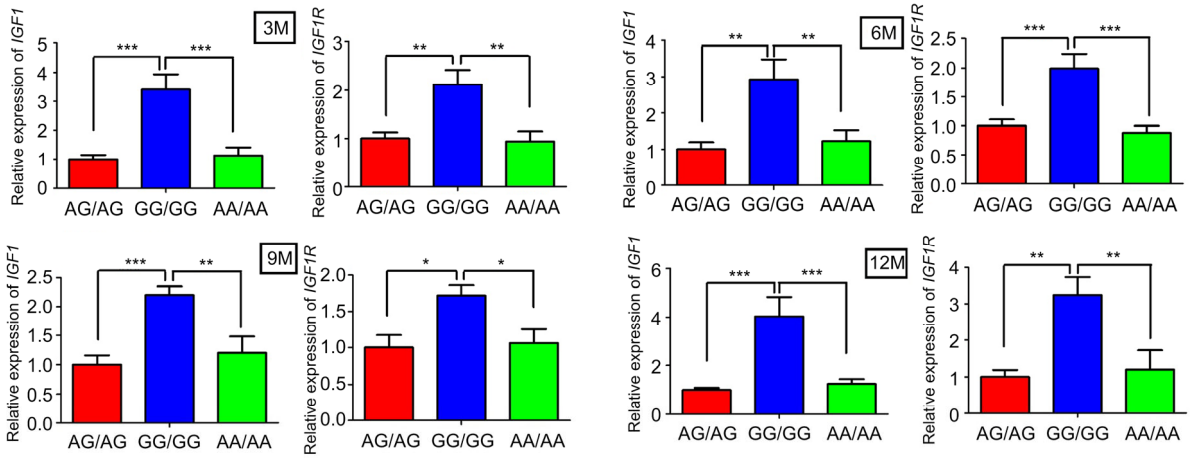

Fig. 2 Real-time quantitative PCR analysis of the expression of *IGF1* and *IGF1R* in semitendinosus muscle of buffalo with the three haplotypes (AG/AG, GG/GG, AA/AA) at ages of 3, 6, 9, and 12 months

Data are expressed as mean±SEM (n=9 in triplicate). The expression level of each target gene in buffaloes with haplotype AG/AG was considered the baseline. * P<0.05, ** P<0.01, and *** P<0.001 after Bonferroni's correction. M: months

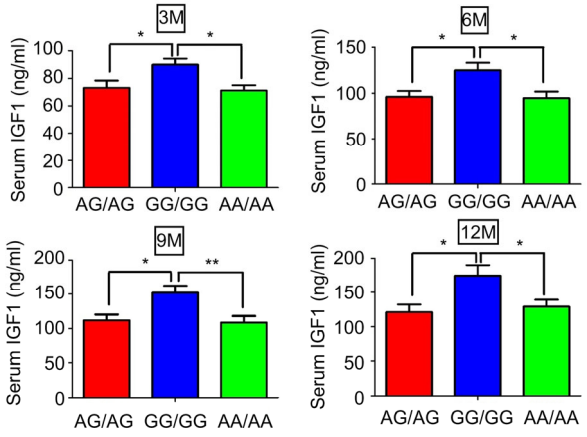

Fig. 3 Serum concentrations of IGF1 in buffaloes with the three haplotypes (AG/AG, GG/GG, AA/AA) at ages of 3, 6, 9, and 12 months (M)

Data are expressed as mean±SEM (n=9 in triplicate). * P<0.05 and ** P<0.01 after Bonferroni's correction. M: months

4 Discussion

As an important member of the group of somatotropin axis genes, *IGF1* is associated with economically important polygenic traits, such as growth and carcass traits. This prompts us to look for polymorphisms in this gene and to study their association with growth traits in Egyptian buffaloes. The main findings are detection of two novel SNPs (G64A and G280A) with GG genotype and GG/GG haplotype buffaloes having significantly superior growth performance, higher expression levels of *IGF1* and *IGF1R* mRNA, as well as higher serum concentration of IGF1 at ages of 3–12 months. The G64A and G280A SNPs are novel as none of them has been found in the *IGF1* of animals studied so far (Dierkes *et al.*, 1999; Ge *et al.*, 2001; Chung and Kim, 2005; Siadkowska *et al.*, 2006; Fatima *et al.*, 2009; Islam *et al.*, 2009; de la Reyna *et al.*, 2010; Mullen *et al.*, 2011). In the present study, three genotypes (GG, AA and AG) were identified in each SNP. In pigs and goats, A/G SNP was determined in *IGF1* exon 4, but in a different position (Wang *et al.*, 2011).

Egyptian buffaloes were in the HWE (P>0.05) for *IGF1.4* locus. This means that the distribution of alleles in this population remains roughly the same from one generation to the next. HWE also means no natural selection, no mutation, no migration, or no genetic drift, and that individuals in the population mate randomly with respect to this locus. Egyptian buffaloes possessed medium polymorphism at *IGF1.1* and *IGF1.4* loci, which indicates moderate genetic diversity within these loci in the analyzed animals. The moderate genetic diversity is suitable as a marker in molecular breeding (Vaiman *et al.*, 1994), so these two SNPs are likely to be useful in breeding programs for Egyptian buffaloes, especially for *IGF1.4* locus which is in HWE. Although the G64A and G280A SNPs are not very close to each other, they were in a

very strong LD (D'=1.0), meaning that these SNPs are co-inherited (dependent) and may have a mutual influence on the growth traits. This prompts us to study the association of the combined genotypes (haplotypes) of both SNPs in addition to individual SNP.

Presence of a synonymous SNP does not mean that the mutation has no effect on the function of the candidate gene or other linked gene(s) in a QTL affecting the studied trait. In agreement with this, previous studies reported a significant association between the synonymous rs29012855 SNP in intron 3 of *IGF1* and the growth traits in cattle (Ge *et al.*, 2001; Islam *et al.*, 2009; de la Reyna *et al.*, 2010; Mullen *et al.*, 2011). Moreover, the G3072A SNP in *IGF2* intron 3 has a significant association with muscle growth (van Laere *et al.*, 2003). Our results also revealed a significant association between the G64A (in the noncoding sequence of exon 1) and G280A (in the noncoding sequence of exon 4) SNPs and growth traits, with GG genotype animals having significantly ($P\leq0.05$) higher BW and ADG than those with other genotypes at ages of 3–6 months in G64A SNP and 6–9 months in G280A SNP. A similar A/G SNP in exon 4 of the goat *IGF1* gene (but in a different position) is associated with production traits (Wang *et al.*, 2011).

In the present study, we found a more significant association between GG/GG, AG/AG, AA/AA haplotypes and growth traits (BW and ADG) at a longer time interval (from 3 to 12 months) than those between individual SNPs. This confirms the importance of studying the inheritance of combined genotypes (haplotypes) rather than individual SNPs. Animals with GG/GG haplotype showed higher growth performance than other buffaloes. This effect is probably due to the positive effect of the G allele in the two SNPs on growth. It is therefore necessary to protect individuals with GG/GG haplotype and to utilize them in MAS for superior growth. Although this significant association was found only in the early growing stages, as there were no significant associations between these haplotypes and growth traits in the late stages of slaughter ages, early selection of animals with GG/GG haplotype can be used for higher carcass yields by MAS in Egyptian buffalo population after providing suitable nutrition and management. It is also likely that IGF1 has a different influence at various stages of growth, because *IGF1* expression is regulated depending on developmental and functional status (Werner *et al.*, 1994). As a finding in support of this, an SNP in the promoter of chicken *IGF1* gene was associated with eggshell weight for the last two periods of egg laying, but not for the previous periods (Nagaraja *et al.*, 2000). We also recently detected two non-synonymous SNPs (C261G/alanine>glycine and G263C/alanine>proline) in exon 21 of the *IGF1R* gene and have found that the haplotype, C261G/G263C, was associated with the ADG during the early stages of life (from birth to 6 months) in Egyptian buffaloes (El-Magd *et al.*, 2013). Our further study focuses on the association of the combined genotypes of both G64A/G280A SNPs of *IGF1* and C261G/G263C SNPs of *IGF1R* with growth traits in Egyptian buffaloes.

The noncoding region of exon 1 and promoter region of the bovine *IGF1* contain binding sites for essential transcription factors which can affect transcriptional efficiency of this and other genes. Consequently, polymorphisms in these regions are positively associated with growth traits, back fat thickness, lean meat yield, and carcass weight in cattle (Siadkowska *et al.*, 2006; Islam *et al.*, 2009; Mullen *et al.*, 2011). In agreement with this, in silico analysis of TFBSs predicted that the G allele of G64A SNP introduced two new TFBSs (Thing1-E47 and Snail) that are cancelled by the A allele. Thing1-E47 is a basic helix loop helix (bHLH) transcription factor localized mainly in smooth muscle and linked to other myogenic factors in the bHLH superfamily (such as Myf) in skeletal muscles (Ho Sui *et al.*, 2007). Although the snail acts as a transcriptional repressor, it has no major role in myogenesis (Norton *et al.*, 2013). In silico analysis also revealed that the A allele of G280A SNP formed two new TFBSs (Hen-1 and Snail), while the G allele introduced AML-1 (RUNX1) TFBS. Recently it has been reported that Runx1 is required for myoblast proliferation during muscle regeneration (Umansky *et al.*, 2015).

Although synonymous SNPs do not change amino acids, they can alter mRNA stability, gene expression level, protein synthesis rate, and protein conformation and function (Komar, 2007). In support of this, we also found a significant association between the two synonymous SNPs (G64A and G280A) and *IGF1* mRNA level in semitendinosus muscle with GG/GG haplotype buffaloes having a higher

expression than other buffaloes. Moreover, GG/GG haplotype buffaloes have a higher serum concentration of IGF1 as compared to other buffaloes included in this study. Similarly, C512T SNP in *IGF1* promoter was shown to be associated with serum levels of IGF1 and postnatal growth in cattle (Maj *et al.*, 2008; Mirzaei *et al.*, 2012). Taken together, it is possible that the significant association between G64A and G280A SNPs of *IGF1* and growth traits resulted from increased expression of the growth axis related gene, *IGF1*, and the circulatory level of the IGF1 protein. IGF1 activates mitogenic proliferation and myogenic differentiation in skeletal muscle (Werner *et al.*, 1994; Oksbjerg *et al*., 2004) and therefore it plays an important role in the growth of many tissues. Thus, the two SNPs detected in the present study may influence IGF1 concentration and consequently, growth performance could be changed in Egyptian buffaloes.

IGF1 exerts its action following binding to its specific receptor (IGF1R). Strikingly, *IGF1R* mRNA was also upregulated in semitendinosus muscle in GG/GG haplotype buffaloes. Therefore, G64A and G280A SNPs may directly or indirectly (through LD with causative variants) change *IGF1* gene expression and IGF1 serum concentration, which is accompanied by upregulated *IGF1R* expression. Future approaches could include entire genes and regulatory regions or whole genome sequencing in independent large populations along with functional genomic analyses in an attempt to identify causative polymorphisms.

5 Conclusions

The main finding of this study was that G64A and G280A SNPs of *IGF1* were significantly associated with BW, ADG, expression levels of *IGF1* and *IGF1R* mRNA, as well as serum concentration of IGF1 in buffaloes at ages from 3 to 12 months with superior G allele, GG genotype, and GG/GG haplotype. These SNPs could be valuable genetic markers for selection of Egyptian buffaloes for better performance in the population.

Compliance with ethics guidelines

Mohammed A. EL-MAGD, Ayman A. SALEH, Abeer A. NAFEAA, Shymaa M. EL-KOMY, and Mohamed A. AFIFI declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：水牛 *IGF1* 基因多态性与其生长性状、血清浓度以及 *IGF1* 和 *IGF1R* 基因表达的相关性研究

目　的：探讨胰岛素样生长因子 1（insulin-like growth factor 1，*IGF1*）基因单核苷酸多态性（SNP）与埃及水牛生长性状的关联性。

创新点：发现 *IGF1* 基因多态性对埃及水牛的生长性状、血液生化指标和基因表达有显著影响，并为埃及水牛的选育提供重要的分子遗传标记。

方　法：以 200 头埃及水牛为试验对象，对其 *IGF1* 基因的多态性进行检测，并分析其与生长性状、血液生理生化指标和基因表达的相关性。

结　论：在水牛 *IGF1* 基因多态性分析中，发现两个新的 SNP 位点（G64A 和 G280A）分别分布在外显子 1 和外显子 4 的非编码区。不同基因型的统计分析表明，G64A 和 G280A 位点的 GG 基因型水牛个体分别在 3~6 月龄和 6~9 月龄的体重（BW）和平均日增重（ADG）均显著高于其他基因型（$P<0.05$）。这两个 SNPs 位点的组合基因型产生了三种单倍体 GG/GG、AG/AG 和 AA/AA。在 3~12 月龄的水牛个体中，单倍体基因型与 BW 和 ADG 存在显著关联（$P<0.0001$）。纯合的 GG/GG 单倍体基因型水牛生长性能优于其他水牛。两个 SNP 位点与半腱肌中 *IGF1* 和 *IGF1R* 的 mRNA 水平以及 IGF1 血清浓度水平相关。此外，GG/GG 单倍体水牛表现出较高的 mRNA 和血清浓度水平。综上所述，这两种 SNP 位点 G64A 和 G280A 可作为埃及水牛生长性状选育的重要分子遗传标记。

关键词：胰岛素样生长因子 1（*IGF1*）；胰岛素样生长因子 1 受体（*IGF1R*）；单核苷酸多态性（SNP）；生长性状；水牛