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REGULAR ARTICLES

Association of a novel SNP in exon 10 of the *IGF2* gene with growth traits in Egyptian water buffalo (*Bubalus bubalis*)

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Abstract Insulin-like growth factor 2 (*IGF2*) plays an important role in muscle growth and it might be used as a marker for the growth traits selection strategies in farm animals. The objectives of this study were to detect polymorphisms in exon 10 of *IGF2* and to determine associations between these polymorphisms and growth traits in Egyptian water buffalo. PCR-single-strand conformation polymorphism (SSCP) and DNA sequencing methods were used to detect any prospective polymorphism. A novel single nucleotide polymorphism (SNP), C287A, was detected. It was a non-synonymous mutation and led to replacement of glutamine (Q) amino acid (aa) by histidine (H) aa. Three different SSCP patterns were observed: AA, AC, and CC, with frequencies of 0.540, 0.325, and 0.135, respectively. Association analyses revealed that the AA individuals had a higher average daily gain (ADG) than other individuals (CC and AC) from birth to 9 months of age. We conclude that the AA genotype in C287A SNP in the exon 10 of the *IGF2* gene is associated with the ADG during the age from birth to 9 months and could be used as a potential genetic marker for selection of growth traits in Egyptian buffalo.

Keywords *IGF2* gene · Egyptian buffalo · Sequencing · SSCP · SNP · Average daily gain

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Introduction

Egyptian water buffaloes (*Bubalus bubalis*) are classified according to their geographical locations and minor phenotypic differences into: Beheri, Baladi, and Saidi (FAODAD-IS 2013; Othman 2012). Until 2011, the Egyptian buffaloes are about 3.8 million, which contribute 19.85 % (395,801 t) of total meat production (FAOSTAT 2011). Buffalo could emerge as a promising alternative to crossbred cattle due to its great adaptability to varied ecological conditions, vital to the lives of small farmers, not only are they draught animals, but they are also a source of meat, horns, skin, and particularly, rich and precious milk that may be converted to creams, butter, yogurt, and many cheeses (Fatima et al. 2009; Michelizzi et al. 2010).

Insulin-like growth factor 2 (*IGF2*) is a member of a family of structurally related polypeptides, the IGF family, which also includes two other ligands (*IGF1* and *IGF2*), three cell membrane receptors, and a number of other associated binding proteins and regulators. The majority of circulating IGF2 is produced by the liver, where its expression is stimulated by growth hormone (Holzenberger et al. 2000; O'Dell and Day 1998; Werner et al. 1994). *IGF2* is an imprinted gene with the parental allele expressed and the maternal allele silenced, but a bi-allelic expression driven by promoters 3 and 4 has been observed in the adult tissues (Braunschweig 2012; Chao and D'Amore 2008; Curchoe et al. 2005).

For its significant role in muscle development, association between *IGF2* polymorphisms and growth traits in animals was extensively studied. In bovine *IGF2*, A C292T SNP in the non-translated exon 2 of the *IGF2* gene at position 8656 was found to be associated with the rib eye area and the carcass fat percent in beef cattle (Goodall and Schmutz 2003, 2007). Four SNPs in bovine *IGF2* associated with progeny carcass conformation and progeny carcass weight were detected by (Berkowicz et al. 2011). Another four SNPs in *IGF2* gene

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were also observed in Qinchuan Chinese cattle and their combined genotypes and haplotypes are associated with the withers' height, body length, chest breadth, and chest body weight (Huang et al. 2014). Furthermore, Sherman et al. (2007) demonstrated association between SNP in *IGF2* with carcass merit in beef cattle, average daily gain (ADG), and feed conversion ratio (FCR). However, some other SNPs were detected in bovine *IGF2* but their association with production traits was not elucidated yet (Curchoe et al. 2005; Flisikowski et al. 2007). In porcine, two SNPs in introns 3 and 7 had significant effect on back fat thickness and lean meat content (Li et al. 2010), a G3072A SNP in intron 3 influenced significantly the intramuscular fat (Oczkowicz et al. 2012). A C > G mutation was detected in the exon 2 in chicken *IGF2* influencing growth and carcass traits (Wang 2005). In addition, the relationship between polymorphism in *IGF2* gene and growth traits in a genetically improved farmed tilapia was reported (Juhua et al. 2010).

To date, no study on associations of the *IGF2* gene with growth traits has been reported in buffalo. The objectives of the present study were to detect *IGF2* polymorphisms, to estimate their allele and genotype frequencies within the exon 10 of the *IGF2* gene, and to determine the effects of these markers on growth traits in Egyptian water buffalo.

Material and methods

This work was reviewed and approved by the Animal Care and Welfare Committee of Kafrelsheikh University, Egypt.

Animal source

This study involved 200 purebred Egyptian water buffaloes kept in a single farm, El-Nataff El-Gidid Experimental Station, located in the Kafrelsheikh Governorate, Egypt. These animals were artificially inseminated by fresh semen collected from buffalo studs on the farm. Animals used in this study were chosen at random. All records of growth traits [body weight and calculated average daily gains (ADG)] were collected from the farm records for statistical analysis.

Muscle biopsies

Skeletal muscle biopsies were collected from the middle gluteal muscle under local anesthesia (2 % lidocaine). The collection site was first shaved and washed thoroughly by antiseptic solution and then a sterilized large (5 mm) Bergstrom biopsy needle was inserted at a constant depth using the percutaneous needle biopsy technique. The muscle sample (~75–110 mg) obtained from each biopsy was snap-frozen in liquid nitrogen and then stored at −80 °C until used for RNA extraction.

Total RNA isolation

Each muscle sample was homogenized and a total RNA was extracted using total RNA purification kit following the manufacturer protocol (Fermentas, #K0731, Thermo Fisher Scientific, USA). The extracted RNA was dissolved in nuclease-free water and the concentration and purity were determined using NanoDrop (UV-vis spectrophotometer Q5000, Quawell, USA) by absorption at 260 and 280 nm. The quality and integrity of total RNA were assessed by inspection of the ribosomal RNA bands (18S and 28S) in ethidium bromide-stained 1 % agarose gels under ultraviolet (UV) light and electrophoresis of the RNA confirmed that it was intact. The RNA samples were stored at −80 °C.

Reverse transcription

Total RNA (5 μg per sample) was reverse transcribed into complementary DNA (cDNA) using Revert Aid H minus Reverse Transcriptase (Fermentas, #EP0451, Thermo Fisher Scientific, USA) and a mix of oligo (dT) (0.5 μg/reaction), 4-μL 5X reaction buffer, 0.5-μg RNase inhibitor, and 2-μL deoxynucleotide triphosphate (dNTP) mix in a 12.5-μL total reaction volume at 42 °C for 60 min. To terminate the reaction, the tubes were heated at 70 °C for 10 min and then were stored at −80 °C.

Polymerase chain reaction

To amplify exon 8–10 of the *Bubalus bubalis IGF2* gene, one pair of polymerase chain reaction (PCR) primer were designed using Primer 5.0 software based on the published nucleotide sequence information of the *Bubalus bubalis IGF2* (GenBank accession FJ032306). The forward primer was ′5CGTGCTGC TATGCTGCTTAC′3 and the reverse primer was ′5GGTGAC TCTTGGCCTCTCTG′3. The PCR was carried out in a reaction volume of 25 μL, containing 1.0-μL cDNA template (approximately 50 ng), 0.5-μL (0.20 mM) dNTP, 2.5-μL buffer, 1.5-μL (2.5 mM) MgCl2, 1.0-μL 10 μmol/L forward primer, 1.0-μL 10 μmol/L reverse primer, 0.5-μL Taq DNA polymerase (5 U/μL, Fermentas, #K1071, Thermo Fisher Scientific, USA), and 17.0 μL nuclease-free water. Thermal cycling parameters were initial denaturation at 94 °C for 5 min, 35 cycles of amplification (94 °C for 30 s for DNA denaturation, 55 °C for 40 s annealing temperatures, extension at 72 °C for 1 min), and final extension at 72 °C for 5 min. The samples were held at 4 °C. PCR products (408 bp) were electrophoresed on 1 % agarose gels using 1X TAE buffer containing 200 ng/mL ethidium bromide.

Single-stranded conformation polymorphism and sequencing

Five-microliter PCR products and 5-μL denaturing solution (25-mM EDTA, 95 % formamide, 0.025 % bromophenol

blue, and 0.025 % xylene cyanole), heated for 10 min at 98 °C, and chilled on ice and then loaded on nondenaturing 12 % polyacrylamide gels (39:1 acrylamide to bis-acrylamide). After electrophoresis, which was performed in 1X Tris–borate–EDTA (TBE) buffer at 150 V for 10–12 h at 4 °C, the DNA fragments in the gel were stained with a 0.5-μg/ml solution of ethidium bromide in 1X TBE buffer for 20 min, and then destained in distilled water for 5 min. Single-stranded conformation polymorphism (SSCP) genotypes were identified by differential migration due to fragment conformation. The PCR products of different electrophoresis patterns were purified and then sent to Macrogen Company (South Korea) to sequence in both directions using ABI 3730XL DNA sequencer (Applied Biosystems, USA). The sequence results were analyzed by Geneious 4.8.4 software.

Statistical analysis

Genotypic and allelic frequencies were directly calculated. Hardy–Weinberg equilibrium (HWE) and population genetic indexes including gene heterozygosity (He), calculated according to Nei (Nei 1973), and effective allele numbers (Ne) were computed by PopGene software (version 1.3) (Yeh et al. 1999). Polymorphism information content (PIC) was calculated according to Botstein et al. (Botstein et al. 1980). The association of the SNP marker genotypes with growth traits was determined using GLM procedures of SPSS (version 16.0). The SNP genotype effects on phenotypic value of each trait were evaluated by the following model: $Y_{klm} = \mu + A_k + G_l + E_{klm}$, where Y_{klm} was the trait measured on each of the klm^{th} animals, μ was the overall mean for each trait, A_k was the fixed effect due to the k^{th} age (in days), G_l was the fixed effect of the l^{th} genotype (AA, AC, and CC genotype) for *IGF2* exon 10, and E_{klm} was the random error.

Results

To detect SNPs in exon 10 of the *IGF2* gene in Egyptian water buffaloes, RNA samples were amplified using RT-PCR and PCR products was 408 bp, subsequently, the PCR fragments were sequenced. The sequences of the *IGF2* exon 10 were submitted to the GenBank database with accession number KC107769. Three different patterns of SSCP were observed, which are designated as AA (1 band), AC (2 bands), and CC (1 band) (Fig. 1a). The nucleotide sequence analyses of these three SSCP patterns revealed the presence of a novel SNP at the nucleotide position of 287 (C to A transition) in exon 10 (Fig. 1b). This novel C287A SNP was non-synonymous and led to substitution of glutamine (Q) amino acid (aa) by histidine (H) aa. The genotype frequencies were 0.540 (108) for AA, 0.325 (65) for AC, and 0.135 (27) for CC with allele frequencies of 0.702 for A and 0.298 for C among the 200 animals that were genotyped for this polymorphism. Chi-Square (χ^2) value was 10.1425 (P value=0.0014). This means that the genotype distributions within buffalo population deviated from HWE ($P<0.05$).

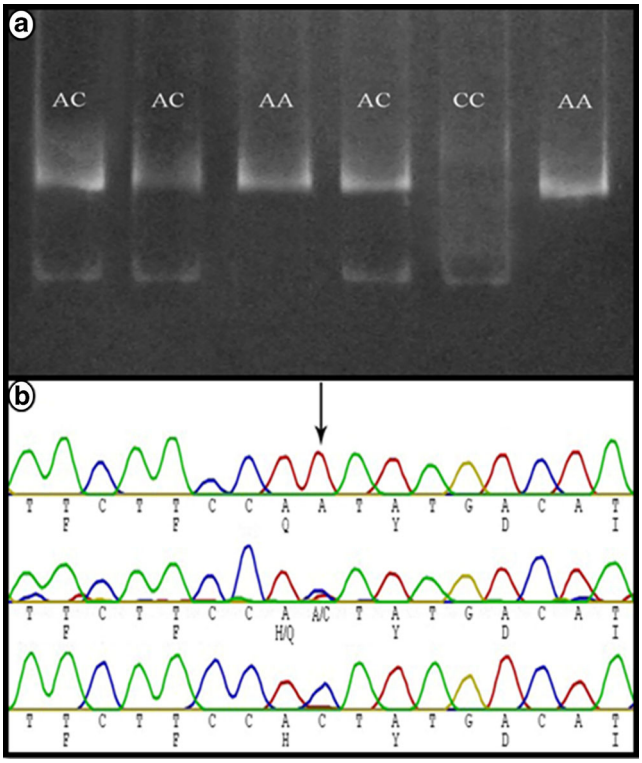

Fig. 1 PCR-SSCP patterns, nucleotide, and amino acid sequences of exon 10 of the *IGF2* gene in Egyptian buffaloes. **a** Three SSCP patterns; genotype AC (*lanes 1*, *2*, and *4*), AA (*lanes 3* and *6*), and CC (*lane 5*). **b** A non-synonymous C287A SNP (*arrow*) was detected in exon 10 of the *IGF2* gene and led to change of glutamine (Q) amino acid (aa) to histidine (H) aa. In all sequences, the amino acid (aa) sequences are below the nucleotides

The population genetic indexes were calculated using the PopGene software (version 1.3) and according to Botstein's method (1980). The value of gene heterozygosity (He) was 0.4180, effective allele numbers (Ne) was 1.7182, and polymorphism information content (PIC) was 0.3308. High heterozygosity in this *IGF2* locus indicated mutation frequencies were high. According to the classification of PIC (low polymorphism if PIC value is <0.25, median polymorphism if 0.25<PIC value <0.5, and high polymorphism if PIC value is >0.5), Egyptian buffaloes possessed median polymorphism at this locus.

Association of AA, AC, and CC genotypes in exon 10 of *IGF2* with the growth traits [body weight and average daily gain (ADG)] were analyzed in Egyptian buffaloes at birth and at 3, 6, 9, 12, 18, and 24 months of age. The ADG of AA genotype animals from birth to 9 months of age ($P\leq0.05$) was significantly higher than animals with AC and CC genotypes (Table 1). Therefore, there was a significant association between the AA genotype and the ADG trait during the early stages of life (from birth to 9 months of age).

Table 1 Association of genotypes (AA, AC, and CC) at exon 10 of *IGF2* gene with growth traits in Egyptian buffalo

Age	Growth traits	Genotype		
		AA	AC	CC
Birth	BW (Kg)	32.66±0.23	32.73±0.24	32.85±0.20
3 months	BW (Kg)	88.62±0.21	82.11±0.24	81.82±0.20
Birth–3 months	ADG (Kg)	0.62±0.01*	0.55±0.02*	0.54±0.04*
6 months	BW (Kg)	161.40±0.27	148.24±0.20	140.50±0.26
3–6 months	ADG (Kg)	0.80±0.02*	0.73±0.03*	0.65±0.05*
9 months	BW (Kg)	212.41±0.21	190.40±0.24	183.70±0.24
6–9 months	ADG (Kg)	0.57±0.04*	0.47±0.03*	0.48±0.07*
12 months	BW (Kg)	258.20±0.22	248.21±0.20	245.15±0.21
9–12 months	ADG (Kg)	0.51±0.05	0.64±0.08	0.68±0.04
18 months	BW (Kg)	334.35±0.50	322.66±0.60	320.96±0.80
12–18 months	ADG (Kg)	0.42±0.07	0.41±0.04	0.42±0.03
24 months	BW (Kg)	417.80±2.09	415.22±2.81	409.82±2.34
18–24 months	ADG (Kg)	0.46±0.05	0.51±0.05	0.49±0.03

Values are least squares means (±SEM)

BW bodyweight; *ADG* average daily gain

*Significance within the same raw at $P \leq 0.05$

Discussion

IGF2 is an important regulator of growth, development, and skeletal muscle differentiation in vertebrates (Bagnicka et al. 2010; Brown et al. 2009; Clemmons 1997; Curi et al. 2005; Duan et al. 2010; Holzenberger et al. 2000). Therefore, *IGF2* gene could be taken as a candidate gene for growth and meat production traits (Carrodeguas et al. 2005; Goodall and Schmutz 2007; Han 2009; Huang et al. 2013; Li et al. 2010; Sherman et al. 2007; Wang 2005; Zhang and Li 2008). We have previously identified polymorphisms in some myogenic genes (e.g., *IGF1R*, *MyoD*, and *MyoG*) and have studied their associations with growth traits in Egyptian buffaloes (El-Magd et al. 2013a, b). In the current study, we detected a novel C287A SNP in the exon 10 of the *IGF2* gene in Egyptian buffalo. This SNP was non-synonymous and led to change of glutamine (Q) aa to histidine (H) aa.

SSCP is technically simple and relatively highly sensitive for the identification of most polymorphisms in a single strand of DNA. PCR-SSCP followed by DNA sequencing were successfully used to detect novel SNPs in the buffalo myogenic genes including *IGF2* (this study), *IGF1R*, *MyoD*, and *MyoG* genes (El-Magd et al. 2013a, b). In the present study, three genotypes (AA, AC, and CC) were identified for C287A SNP in Egyptian buffaloes. All genotyped Egyptian buffaloes (n=200) deviated from HWE (P<0.05), suggesting a change in the distribution of alleles from one generation to the next. This deviation may be due to heterozygote deficiency or homozygous excess or small sample sizes.

Genetic diversity is essential for the preservation of adaptive potential of species and the improvement of production of potentially highly selected animals. According to the classification of PIC (low polymorphism if PIC value is <0.25, median polymorphism if 0.25<PIC value<0.5, and high polymorphism if PIC value is >0.5) (Botstein et al. 1980), Egyptian buffaloes had median polymorphism at *IGF2* locus. This reflected that there was a moderate genetic diversity within this locus in the analyzed animals.

The association between SNPs in *IGF2* and growth traits was reported in many animals. In cattle *IGF2*, a T/G transition in exon 9 affects body mass and daily weight gain (Zhao et al. 2002), and a T150C SNP in exon 2 affects rib-eye area and body weight measurements (Schmutz and Goodall 2005; Sherman et al. 2007). This were also reported in pig, chicken, and fish (Wang 2005; Juhua et al. 2010). In consistence, statistical analysis revealed a significant association between C287A SNP and average daily gain (ADG) from birth to 9 months of age in Egyptian buffaloes. AA genotype animals have a higher ADG than the other two genotypes (AC and CC). This suggested that the genotype AA should be used as molecular markers in the future for the selection of greater ADG in Egyptian buffaloes. Similar effects have been previously reported by us for another gene of IGF family, the *IGF1R* gene, where two non-synonymous mutations produced by C261G and G263C SNPs in exon 21 were shown to be associated with the ADG during the early stages of life (from birth to 6 months of age) of Egyptian buffalo (El-Magd et al. 2013a). Because exons 8–10 encode pre-proprotein of IGF2 (Goodall and Schmutz 2007), and the detected SNP was present at exon 10 so it may affect the function of the protein. However, further investigations are recommended to validate the effect of this SNP on the gene expression of *IGF2* and other related genes such as *IGF1*, *IGF1R*, and *IGF2R*.

The phenotype effect of a single SNP may be influenced by other SNPs in other loci in the same gene or other linked genes especially in polygenic traits such as growth traits. This agrees with the conclusion that the inheritance of haplotype combinations was more effective than that of a single SNP (Fallin

et al. 2001). It is interesting to study the association of the combined genotypes/haplotypes of both C287A SNP in exon 10 of *IGF2* and C261G/G263C SNPs in exon 21 of the *IGF1R* with growth traits in Egyptian buffaloes.

Conclusion

The major finding of this study was that the novel C287A SNP in exon 10 of *IGF2* is associated with ADG in Egyptian buffaloes. This suggests that this SNP may be used as a genetic marker which could be used for the marker-assisted selection in breeding programs for Egyptian buffaloes. However, further investigations on more Egyptian buffalo populations are needed to verify the effects of this SNP, as well as the effect of the other DNA polymorphisms in this gene and other linked loci.

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Conflict of interest This manuscript has no conflicts of interest.

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