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Effects of a novel SNP of *IGF2R* gene on growth traits and expression rate of *IGF2R* and *IGF2* genes in gluteus medius muscle of Egyptian buffalo

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ABSTRACT

Insulin-like growth factor 2 receptor (*IGF2R*) is responsible for degradation of the muscle development initiator, *IGF2*, and thus it can be used as a marker for selection strategies in the farm animals. The aim of this study was to search for polymorphisms in three coding loci of *IGF2R*, and to analyze their effect on the growth traits and on the expression levels of *IGF2R* and *IGF2* genes in the gluteus medius muscle of Egyptian buffaloes. A novel A266C SNP was detected in the coding sequences of the third *IGF2R* locus (at nucleotide number 51 of exon 23) among Egyptian water buffaloes. This SNP was non-synonymous mutation and led to replacement of Y (tyrosine) amino acid (aa) by D (aspartic acid) aa. Three different single-strand conformation polymorphism patterns were observed in the third *IGF2R* locus: AA, AC, and CC with frequencies of 0.555, 0.195, and 0.250, respectively. Statistical analysis showed that the homozygous AA genotype significantly associated with the average daily gain than AC and CC genotypes from birth to 9 mo of age. Expression analysis showed that the A266C SNP was correlated with *IGF2*, but not with *IGF2R*, mRNA levels in the gluteus medius muscle of Egyptian buffaloes. The highest *IGF2* mRNA level was estimated in the muscle of animals with the AA homozygous genotype as compared to the AC heterozygotes and CC homozygotes. We conclude that A266C SNP at nucleotide number 51 of exon 23 of the *IGF2R* gene is associated with the ADG during the early stages of life (from birth to 9 mo of age) and this effect is accompanied by, and may be caused by, increased expression levels of the *IGF2* gene.

1. Introduction

Egyptian buffalo belongs to the river buffalo (*Bubalus bubalis*) and is classified into Baladi, Beheri, Menoufi and Saidi according to their geographical locations and some phenotype differences; however, PCR-RAPD technique showed that no significant genetic difference among Egyptian buffaloes (FAODAD-IS, 2013; Othman, 2012). Buffalo finds great interest from farmers in Egypt due to its great economic importance. Buffalo can utilize protein and dietary fiber more efficiently than cattle (Michelizzi et al., 2010; Paul et al., 2003). Moreover, buffalo meat contains less intramuscular fat, cholesterol, calories and more protein than beef so it is healthier than beef (Khedkar et al., 2003; Nanda and Nakao, 2003; Ranjhan, 2007; Valin et al., 1984) and more acceptable for consumption (Spanghero et al., 2004).

The bovine insulin-like growth factor 2 receptor (*IGF2R*) gene is localized on chromosome 9 (BTA9) (Berkowicz et al., 2012; Friedl and Rottmann, 1994), on the telomeric end (regions 27–28) in the vast majority of metaphases (Friedl and Rottmann, 1994). It spans approximately 101 kb and consists of 48 translated exons (Berkowicz et al., 2012) and approximately 9.5 kb mRNA (Lobel et al., 1987). The bovine IGF2R is a type-I transmembrane glycoprotein consisting of four structural regions: a 163-residue C-terminal cytoplasmic region, a single 23-residue transmembrane region, a 44-residue amino-terminal signal sequence, and a 2269-residue extracellular N-terminal region consisting of 15 homologous repeats (domains) of approximately 147 amino acids each (16%–38% identity) (Kornfeld, 1992; Lobel et al., 1987). Each repeat also contains a highly conserved 13-amino acid unit bordered by cysteine residues that may be functionally important. The extracytoplasmic region of IGF2R has three binding sites: one at domain 11 for IGF2 and two at 3, 5 and 9 domains for Man-6-P (Dahms et al., 1994; Reddy et al., 2004; Williams et al., 2007). The binding of IGF2 with IGF2R causes lysosomal degradation of IGF2. Subsequently, IGF2R serves to clear IGF2 from the circulation or degrade excess circulating IGF2 (Farmer et al., 2013; Kornfeld, 1992; Spicer and Aad, 2007). The soluble IGF2R affects the size of some organs exclusively by reducing the biological activity of IGF2 (Zaina and Squire, 1998).

Abbreviations: aa, amino acid; ADG, average daily gain; He, gene heterozygosity; Ho, gene homozygosity; HWE, Hardy–Weinberg equilibrium; IGF2, insulin-like growth factor 2; IGF2R, insulin-like growth factor 2 receptor; LSM, least square means; Man-6-P, mannose 6 phosphate; mo, month; Ne, effective allele numbers; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; SNP, single nucleotide polymorphism; SSCP, single-strand conformation polymorphism; χ^2, Chi-square value.

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As a result of its physiological action as a negative regulator for *IGF2*, there are some published studies on association between *IGF2R* polymorphisms and growth traits. A total of 9 SNPs were identified in the human *IGF2R* gene (Neuvians et al., 2005). Of them, the non-synonymous C901G SNP in exon 6 was associated with birth weight (Kaku et al., 2007). Another SNP, C8191754G, in maternal *IGF2R* was also associated with birth weight with the homozygotes CC individuals had the highest birth weight (Garmroudi et al., 1996). In bovine, T65037T SNP in *IGF2R* was associated with carcass weight (Berkowicz et al., 2012). Some other SNPs were detected in *IGF2R* but their association with growth traits was not studied; g.C86262T and g.G86531A SNPs in intron 36 of bovine *IGF2R* (Magee et al., 2010), G384A SNP in exon 48, G5403C and G5421T SNPs in exon 37 of pig *IGF2R* (Ludwig et al., 1996; Van Laere et al., 2003), as well as G7768T SNP and A8593G indel in the 3′UTR of dog *IGF2R* (Bonafe et al., 2003). To date, no study on associations of the *IGF2R* gene with growth traits has been reported in buffalo.

Their roles in muscle growth and development make *IGF2R* and *IGF2* genes potential candidates for molecular markers of meat production and growth traits in livestock. However the polymorphisms of *IGF2R* gene and their potential effect on *IGF2R* and *IGF2* gene expression levels and muscle growth and development have not elucidated yet.

Thus, the objective of this study was to identify polymorphisms in three coding loci of *IGF2R*, and to investigate their possible effect on the growth traits and *IGF2R* and *IGF2* mRNA expression levels in the gluteus medius muscle of Egyptian buffaloes.

2. Material and methods

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

2.1. Animal source

Animal source was from El-Nataff El-Gidid Experimental Stations, Mahalet Mousa, Kafrelsheikh Governorate. The 200 animals used in this study were chosen at random. These animals were pure Egyptian water buffaloes, based on farm records and animal appearance. Moreover, these animals were artificially inseminated by fresh semen collected from buffalo studs on the farm. All records of body weight and average daily gain for different growth periods (at birth and at 3, 6, 9, 12, 18, and 24 mo of age) were collected from the farm records.

2.2. Muscle biopsies

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

2.3. Total RNA isolation

After homogenization of muscle samples, a total RNA was extracted using easy-RED™ following the manufacturer's protocol (iNtRON Biotechnology, #17063, Korea). The concentration and purity of the extracted RNA 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.

2.4. One step reverse transcription polymerase chain reaction (RT-PCR)

Amplification of the *IGF2R*, *IGF2* and *β.actin* were performed by using primers (Table 1) which were designed using Primer 5.0 software based on the published nucleotide sequence information of the *B. bubalis* (GenBank accession FJ032306 for *IGF2*), and the *Bos taurus* (GenBank accession NM_174352 for *IGF2R*, and NM_173979 for *β.actin*). The RT-PCR was carried out as described by the manufacturer (Jena Bioscience, #PCR-509S). In brief, 10 μL RNA/primer mix was first prepared by adding 2.0 μL RNA template (approximately 1 μg), 1.0 μL 10 μmol/L forward primer, 1.0 μL 10 μmol/L reverse primer and 6.0 μL nuclease free water and then the mix was incubated at 70 °C for 5 min followed by 5 min incubation at room temperature. Subsequently, 40 μL RT-PCR mix was prepared by adding 25 μL of SCRIPT RT-PCR Reaction Mix [containing SCRIPT Reverse Transcriptase, Hot Start Polymerase and RNase Inhibitor in storage buffer with 50% glycerol (v/v)], 2 μL of SCRIPT RT-PCR Enzyme Mix (contain 2× Reaction Buffer and dNTPs) and 13.0 μL of RNase-free water. The two tubes (40 μL RT-PCR mix and 10 μL RNA/primer mix) were mixed together, incubated at 50 °C for 60 min and then put in the Thermal Cycler (Techne TC-3000, USA) with the following thermal cycling conditions: initial denaturation at 94 °C for 5 min, 35 cycles of amplification (DNA denaturation at 94 °C for 40 s, annealing at 55–59 °C for 1 min (Table 1), extension at 72 °C for 1 min) and final extension at 72 °C for 10 min. RT-PCR products were electrophoresed on 1.5% agarose gels using 1× TAE buffer containing 200 ng/mL ethidium bromide. DNA bands were visualized using long wavelength UV illumination and photographed using a gel documentation system (UV products, Ltd, Cambridge, UK). The level of expression of different bands was analyzed by an ImageJ gel analysis program (Abramoff et al., 2004). This relies on comparing the density of each target gene band with the corresponding control band relative to positive control *β.actin* band.

2.5. SSCP and sequencing

For SSCP analysis, the PCR product (5 μL) was mixed with an equal volume of denaturing solution (25 mM EDTA, 95% formamide, 0.025% xylene-cyanol and 0.025% bromophenol blue), heated for 5 min at 94 °C, rapidly chilled on ice and then loaded on a nondenaturing 12% polyacrylamide gel (39:1 acrylamide to bis-acrylamide). SSCP gel was run in 1× TBE buffer at 200 V for 10 h at 4 °C. To visualize the DNA

Table 1
Forward and reverse primer sequences for the coding regions of *IGF2R*, *IGF2*, and *β.actin* genes, annealing temperatures (Ta), size of PCR products (bp), and localization of the representative parts of these regions.

Locus	Forward primer (′5 ——— ′3)	Reverse primer (′5 ——— ′3)	Ta (°C)	Size (bp)	Coding region
IGF2R.1	ATATGGGTGTTGCCCAGTGT	GCCCCTTCCTTCACGTAACT	57	589	Exons 2–7
IGF2R.2	TTGATCTATTTCGGGGGTGA	CTTGTAGGCGTCCTTCTTCG	56	717	Exons 10–15
IGF2R.3	CATCGGGAAGACGTTTCTGT	GTCGTTCTGGAGCTGAAAGG	59	736	Exons 21–26
IGF2	CGTGCTGCTATGCTGCTTAC	GGTGACTCTTGGCCTCTCTG	55	408	Exons 8–10
β.actin	CGACAACGGCTCCGGCATGT	CTCCTCAGGGGCCACACGGA	58	271	Refer to NM_173979

fragments, the gel was stained with a 0.5 μg/mL solution of ethidium bromide in 1× TBE buffer for 20 min, and then destained in distilled water for 15 min. SSCP genotypes were identified by differential migration due to fragment conformation. After the polymorphism was detected, 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 Biosystem, USA). The sequences were analyzed with Geneious 4.8.4 software.

2.6. Statistical analysis

Allele and genotype frequencies of A266C SNP at nucleotide number 51 of exon 23 in buffalo *IGF2R* were estimated by direct counting. Hardy–Weinberg equilibrium (HWE), *He* (gene heterozygosity), *Ho* (gene homozygosity), and *Ne* (effective allele numbers; reciprocal of homozygosity) were computed by POPGENE software (Version 1.31; Yeh et al., 1999). Association analyses were conducted using least square means (LSM) estimates procedure by using SPSS (version 16.0) software to analyze the relationship between the three genotypes (CC, CA and AA) of A3327C SNP and growth traits in buffalo according to the following linear model: $Y_{klm} = \mu + A_k + G_l + E_{klm}$, where Y_{klm} was the trait measured on each of the klmth animals, μ was the overall population mean, A_k was fixed effect due to the kth age (in days), G_l was the fixed effect associated with lth genotype and E_{klm} was the random error.

The band intensities from each gene were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The data were statistically analyzed by using GraphPad prism® Version 5.0 software (San Diego, CA, USA). The statistical significance of each parameter among groups was evaluated by Student's t-test or the Mann–Whitney U-test (for nonparametric values). *P*-values of <0.05 were considered statistically significant.

3. Results

The three coding loci of the *IGF2R* gene (*IGF2R.1*, *IGF2R.2* and *IGF2R.3*) were determined using RT-PCR (Fig. 1A) and their genotyping was identified in 200 Egyptian buffaloes using SSCP method. Three SSCP banding patterns (AA/AC/CC) were detected in the coding region of *IGF2R.3* locus (Fig. 1B). The sequences of *IGF2R.1*, *IGF2R.2* and *IGF2R.3* in Egyptian water buffalo were submitted to GenBank with accession numbers KC107775, KC415273 and KF031126 respectively, which showed a novel non-synonymous A266C SNP at nucleotide number 51 of exon 23 of *IGF2R.3* locus among the Egyptian buffaloes (Fig. 1C) and 43 SNPs as compared to *B. taurus* (FJ032307) (S1).

The frequencies of the AA, CA, and CC genotypes in the A266C SNP of the *IGF2R.3* locus were 0.555, 0.195 and 0.250, respectively. Frequencies of the A and C alleles were 0.6525 and 0.3475 respectively. The three genotypes were in Hardy–Weinberg disequilibrium ($P < 0.05$), as shown in Table 2. The values of the difference between expected and observed *He* were approaching 0.5. The values of *Ne* were approaching 2 (Table 2).

Association of AA, AC, and CC genotypes at A266C SNP at nucleotide number 51 of exon 23 of *IGF2R* with the growth traits (body weight and average daily gain, ADG) was analyzed in Egyptian buffaloes at birth and at 3, 6, 9, 12, 18, and 24 mo of age. There was a significant association between ADG and A266C from birth to 9 mo of age ($P \leq 0.05$), with a significantly high ADG of the AA individuals (Table 3). Therefore the homozygous AA genotype is more favorable than the heterozygous AC or homozygous CC genotype.

To identify whether this increase in ADG is accompanied by change in *IGF2R* gene expression levels, semi-quantitative RT-PCR was used to estimate the expression level of *IGF2R* in the gluteus medius muscle of high (AA) and low (AC, CC) ADG Egyptian buffaloes from birth to 9 months of age. No significant change in *IGF2R* mRNA levels was observed in high and low ADG animals (Fig. 2).

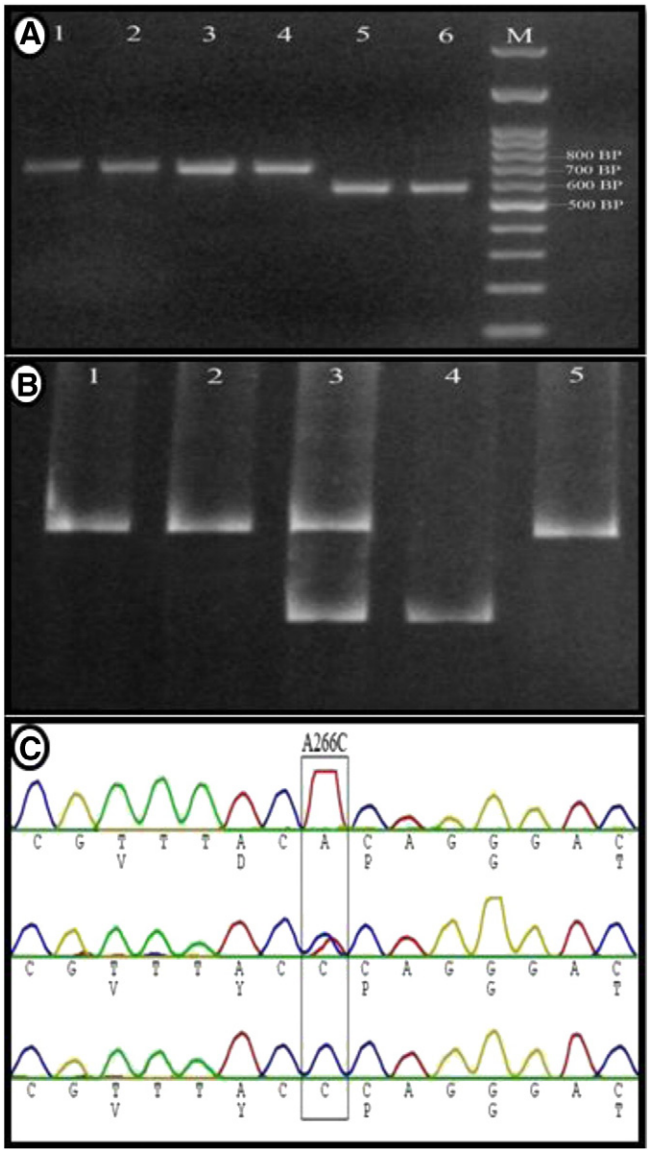

Fig. 1. RT-PCR products of *IGF2R.1–3* loci (A), PCR-SSCP patterns (B), and nucleotide and amino acid sequences of *IGF2R.3* locus (C) in Egyptian buffalo, showing the genotypes and polymorphisms. (A) Ethidium bromide stained agarose gel of RT-PCR products from 6 different samples: 589 bp *IGF2R.1* (lanes 5, 6), 717 bp *IGF2R.2* (lanes 3, 4), and 736 bp *IGF2R.3* (lanes 1, 2). (B) Three SSCP patterns from 5 different samples; genotypes AA (lanes 1, 2, 5), AC (lane 3) and CC (lane 4). (C) One non-synonymous A266C SNP was detected in *IGF2R.3* locus which AA (aspartic acid), AC (aspartic and tyrosine) and CC (tyrosine). The amino acid sequences are below the nucleotides and the box indicates the position of the polymorphism.

To check whether the increased ADG can change the level of *IGF2* gene expression, the level of *IGF2* mRNA in the gluteus medius muscle of high (AA) and low (AC, CC) ADG Egyptian buffaloes from birth to 9 months of age was estimated. Expression analysis showed increased level of *IGF2* mRNA after birth and at 3 months of age (Fig. 3) as well as at 6 and 9 months of age (Fig. 4). The highest *IGF2* mRNA level was estimated in the muscle of animals with the AA homozygous genotype as compared to the AC heterozygotes and CC homozygotes.

4. Discussion

One of the most important goals of animal breeding program is to get animal with increased growth rate. To achieve this, the growth related genes, and their polymorphisms, should be first identified. Advances

Table 2
Genotypic and allelic frequencies, value of χ^2 test, and diversity parameter of A266C SNP of buffalo *IGF2R* gene.

SNP	Genotype frequencies (number)			Allele frequencies		χ^2 (HWE)	*P* value	*He*	*Ne*
	AA	AC	CC	A	C				
A3327C	0.555 (111)	0.195 (39)	0.250 (50)	0.6525	0.3475	65.6207	0.000 (<0.05)	0.4535	1.8298

χ^2: Chi-square value.
HWE: Hardy–Weinberg equilibrium.
He: gene heterozygosity.
Ne: effective allele numbers.

in molecular genetics have resulted in the identification of genes and their polymorphisms which influence meat production and growth traits in animals. *IGF2R* gene encodes a multi-functional protein that has been implicated in regulation of cell growth and apoptosis (Chen et al., 2004). It also mediates trafficking of mannose-6-phosphate (M6P)-containing proteins and turnover of the mitogenic hormone IGF2 (Brown et al., 2009; Dindot et al., 2004; Meitern et al., 2013; Williams et al., 2007). IGF2R is involved in fetal and early neonatal development (Friedl and Rottmann, 1994) and an indicator of postnatal fitness (Suteevun-Phermthai et al., 2009). Therefore, *IGF2R* has been suggested as an excellent candidate gene for genetic variation in growth traits in vertebrates (Berkowicz et al., 2012; Garmroudi et al., 1996; Kaku et al., 2007; Neuvians et al., 2005). In this study, 3 loci of the buffalo *IGF2R* coding region were chosen to test their association with growth traits in Egyptian water buffalo. According to the published data, this is the first study that showed the association between coding regions of *IGF2R* and growth traits in buffalo.

Knowledge on *IGF2R* gene polymorphism is limited, and little is known about its effect on growth and muscle development in farm animals. Few SNPs in the *IGF2R* gene, which have been associated with growth traits were identified in different vertebrates (Berkowicz et al., 2012; Garmroudi et al., 1996; Kaku et al., 2007; Neuvians et al., 2005). In the present study, sequence analysis of all examined Egyptian buffaloes has showed a novel A266C SNP at *IGF2R.3* locus (equivalent to nucleotide number 51 of exon 23 of *IGF2R*). This SNP was non-synonymous mutation and led to replacement of Y (tyrosine) amino acid (aa) by D (aspartic acid) aa. Moreover, comparing the sequences of three loci of Egyptian buffalo *IGF2R* with the corresponding sequences in *B. taurus* (GenBank accession FJ032307) revealed 43 SNPs; 12 non-synonymous and 31 synonymous.

All examined buffaloes deviated from HWE ($P < 0.05$) for this A266C SNP. In general, deviation from the HWE proportions suggests that at least one of the standard underlying assumptions for the test (non-overlapping generations, large population size with random mating, no mutation, no migration, and no selection) may be violated. In this study, HWE deviation may be due to small sample size (only 200 animals). This indeed is accompanied by heterozygote deficiency (AC genotypes = 0.195) and homozygous excess (AA and CC genotypes = 0.805).

Previous studies have identified SNPs in the coding regions of the *IGF2R* gene that show considerable associations with growth traits in humans (Garmroudi et al., 1996; Kaku et al., 2007; Neuvians et al., 2005) and bovines (Berkowicz et al., 2012). Our results also reported significant association between A266C SNP of the *IGF2R* gene and ADG in Egyptian water buffalo. Animals with the homozygous AA genotype showed higher ADG values than animals with the heterozygous AC and the homozygous CC genotypes, which suggested that the genotype A266C SNP — AA should be used as a molecular marker in the future for the selection of ADG in buffalo. The above results suggested that the buffaloes with A266C SNP — AA genotypes could be selected to obtain greater ADG at early stage of life. This SNP affected the buffalo early growth from birth to 9 mo of age, which is consistent with reports on humans and mice. Previous studies on humans indicated that there were significant associations of the *IGF2R* gene with growth and development during early stages of life (Garmroudi et al., 1996; Kaku et al., 2007; Rezgui et al., 2009). In mice, expression of *IGF2R* affects an increase in intrauterine growth only shortly before birth (Wutz et al., 2001). Similar effects have been previously reported by us for another gene of the 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 mo of age) of Egyptian buffalo (El-Magd et al., 2013).

Relative proportion of IGF1R and IGF2R levels influences the relative maximal effects of IGF2 versus IGF1, which may be affected by cell type and/or species (Spicer and Aad, 2007). Mammalian IGF2R has a higher affinity about 100 times for IGF2 than IGF1 (Kornfeld, 1992), binds two distinct ligands, mannose 6-phosphate and insulin like IGF2 with high affinity (Dahms et al., 1994). Previous studies have shown that

Table 3
Association of genotypes at *IGF2R.3* locus with growth traits in Egyptian buffalo.

Age	Growth traits	Genotype			*P* value
		AA	AC	CC	
Birth	BW (kg)	32.57 ± 0.24	32.76 ± 0.22	32.88 ± 0.23	0.2353
3 mo	BW (kg)	87.65 ± 0.23	81.13 ± 0.20	80.80 ± 0.23	0.0547
Birth–3 mo	ADG (kg)	0.61 ± 0.02*	0.54 ± 0.03*	0.53 ± 0.02*	0.0359 (<0.05)
6 mo	BW (kg)	160.4 ± 0.28	147.26 ± 0.24	142.3 ± 0.27	0.1197
3–6 mo	ADG (kg)	0.81 ± 0.01*	0.73 ± 0.02*	0.68 ± 0.02*	0.0426 (<0.05)
9 mo	BW (kg)	211.60 ± 0.24	191.40 ± 0.22	182.60 ± 0.25	0.3167
6–9 mo	ADG (kg)	0.57 ± 0.03*	0.49 ± 0.06*	0.45 ± 0.05*	0.0328 (<0.05)
12 mo	BW (kg)	253.23 ± 0.22	246.31 ± 0.24	245.15 ± 0.24	0.4660
9–12 mo	ADG (kg)	0.46 ± 0.03	0.61 ± 0.07	0.69 ± 0.05	0.4862
18 mo	BW (kg)	332.85 ± 0.70	323.70 ± 0.65	327.91 ± 0.80	0.3251
12–18 mo	ADG (kg)	0.44 ± 0.04	0.43 ± 0.05	0.46 ± 0.02	0.2696
24 mo	BW (kg)	415.83 ± 2.02	416.37 ± 2.84	410.85 ± 2.67	0.5075
18–24 mo	ADG (kg)	0.46 ± 0.06	0.51 ± 0.09	0.46 ± 0.02	0.2503

BW — bodyweight, ADG — average daily gain.
Values are least squares means (±SEM).
* Significance at $P < 0.05$.

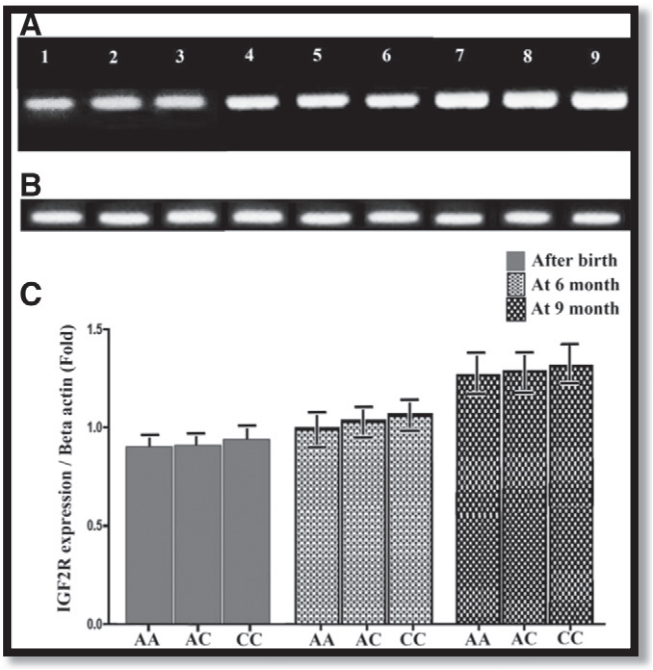

Fig. 2. Gene expression levels of *IGF2R.3* locus containing the A266C SNP in the gluteus medius muscle of Egyptian buffaloes with the three genotypes (AA, AC, CC) from birth to 9 months of age. A–B) Ethidium bromide stained agarose gel of RT-PCR products of *IGF2R.3* locus with size of 736 bp (A) compared to the house keeping gene, *β.actin*, with size of 271 bp (B): lanes 1–3 (after birth), lanes 4–6 (at 6 months of age), and lanes 7–9 (at 9 months of age). C) Band intensity was quantified using ImageJ software and the ratio of *IGF2R* to *β.actin* was calculated. Mean ratios of four samples of three genotypes performed on different samples and data expressed as the mean ± S.E.M. are represented in this figure, relative to the mean ratio of buffaloes after birth. No significant change in *IGF2R* gene expression levels in the gluteus medius muscle was noticed among the three genotypes at 6 or 9 month old Egyptian buffaloes ($P \geq 0.05$).

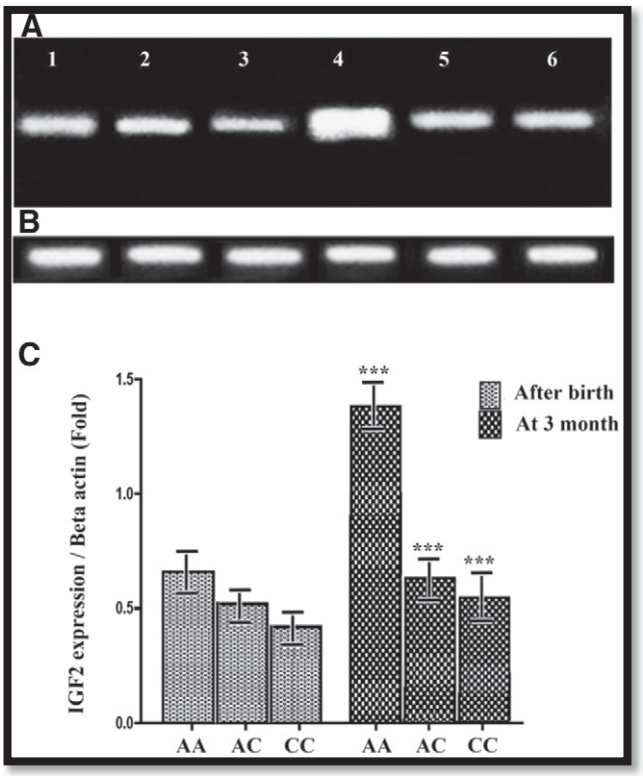

Fig. 3. Gene expression levels of *IGF2* in the gluteus medius muscle of Egyptian buffaloes with the three genotypes (AA, AC, CC) of *IGF2R* A266C SNP after birth and at 3 months of age. A–B) Ethidium bromide stained agarose gel of RT-PCR products of *IGF2* with size of 408 bp (A) compared to the house keeping gene, *β.actin*, with size of 271 bp (B): lanes 1–3 (after birth) and lanes 4–6 (at 3 months of age). C) Band intensity was quantified using ImageJ software and the ratio of *IGF2* to *β.actin* was calculated. Mean ratios of four samples of three genotypes performed on different samples and data expressed as the mean ± S.E.M. are represented on this figure, relative to the mean ratio of the AA after birth. *IGF2* gene expression levels were significantly higher in the gluteus medius muscle of Egyptian buffaloes with genotype AA at 3 months of age. ***Denotes significant difference from buffaloes with genotype AA after birth ($P \leq 0.05$).

IGF2R can titrate extracellular levels of IGF2, and so regulate the growth-promoting function of IGF2 (Wutz et al., 2001). Mouse mutants inheriting maternally a targeted disruption of the imprinted *IGF2R* gene, showed an increase in tissue and serum levels of IGF2 with 135% overgrowth of normal birth weight (Dindot et al., 2004; Meitern et al., 2013). In addition, *IGF2R* null mice were 25–30% larger than their normal siblings and had an elevated level of circulating IGF2 (Lau et al., 1994; Wang et al., 1994). Similarly, disruption of *IGF2R* expression in sheep causes impairment of clearance of IGF2 from the circulation causing fetal overgrowth (Xi et al., 2012). Consistent with this, overexpression of *IGF2R* decreased organ size (Zaina and Squire, 1998). In addition, *IGF2R* gene expression was associated with lean growth which affected cell growth, protein synthesis, and cell proliferation pathways (Williams, 2008). Taken together, *IGF2R* is a negative regulator of growth. Subsequently, any potential polymorphisms in domain 11 (mainly exon 34), which is the specific binding site of IGF2R for IGF2, may account for the correlation with growth in early stages of life (Garmroudi et al., 1996). In contrast, Rezgui et al (2009) studied the correlation between A5002G SNP in exon 34 of domain 11 with impaired childhood growth and found that this Gly1619Arg non-synonymous mutation has no direct effect on receptor function. They suggested that the function of IGF2R depends on another domain that is remotely located with respect to 1619.

The association between A266C SNP and the increased ADG may be followed by, or correlated to, change in the expression level of the *IGF2R* gene. In contrast, no significant change was observed in gene expression of the *IGF2R* locus containing A266C SNP in animals with high (AA genotype) and low (AC, CC genotypes) ADG. On the other hand, the gene expression of the *IGF2* was significantly up-regulated in high ADG animals (compared to low ADG animals). Therefore, it is possible that change of tyrosine to aspartic acid aa, due to A266C SNP, may directly or indirectly change receptor function and so decrease degradation of IGF2. Although, A266C SNP is located in exon 23 (domain 7) remote from the specific binding site (domain 11), it is possible that the conformation of the IGF2R protein may be changed by this SNP which in turn can affect its binding with IGF2. Further investigations using yeast two hybrid or co-immunoprecipitation experiments can confirm this possibility. Thus, the increase ADG in AA genotype animals is likely to be secondary to the increase of IGF2 activity due to change in IGF2R function. Further investigations including gene and protein disruptions of IGF2R are required to clarify the actual mechanism by which the A266C SNP can increase the ADG.

5. Conclusion

This association study along with quantification of the mRNA expression experiments on *IGF2R* and *IGF2* may provide some details about the molecular mechanism underlying the effect of the detected *IGF2R* SNP on growth traits in buffalo. Further research and validation of the various allelic effects, and functional mechanisms are needed before claiming that the identified IGF2R SNP can be used as a marker useful to selection strategy of good meat producer animal.

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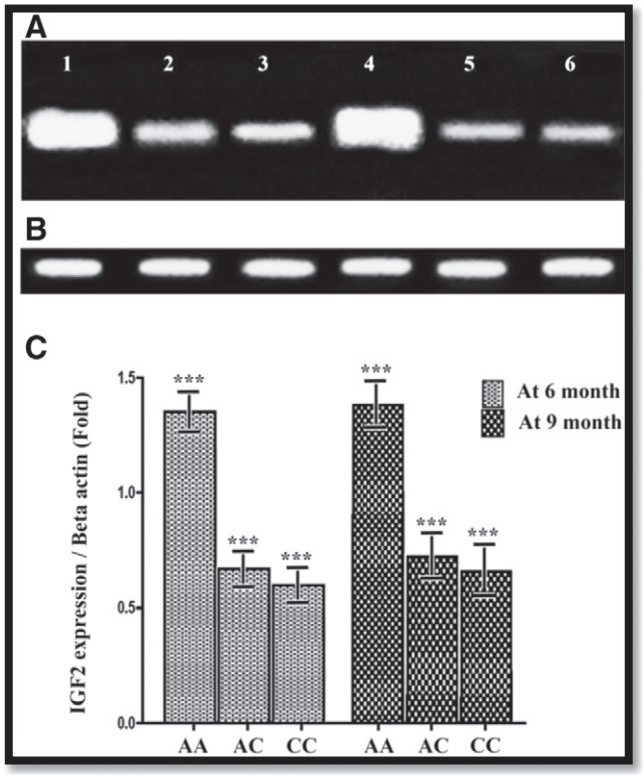

Fig. 4. Gene expression levels of *IGF2* in the gluteus medius muscle of Egyptian buffaloes with the three genotypes (AA, AC, CC) of *IGF2R* A266C SNP at 6 and 9 months of age. A–B) Ethidium bromide stained agarose gel of RT-PCR products of *IGF2* with size of 408 bp (A) compared to the house keeping gene, *β.actin*, with size of 271 bp (B): lanes 1–3 (at 6 months of age) and lanes 4–6 (at 9 months of age). C) Band intensity was quantified using ImageJ software and the ratio of *IGF2* to *β.actin* was calculated. Mean ratios of four samples of three genotypes performed on different samples and data expressed as the mean ± S.E.M. are represented on this figure, relative to the mean ratio of the CC at age of 6 months. *IGF2* gene expression levels were significantly higher in the gluteus medius muscle of Egyptian buffaloes with genotype AA at age of 6 and 9 months. ***Denotes significant difference from buffaloes with genotype AA after birth ($P \leq 0.001$).

Conflict of Interest statement

None.

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