Molecular isolation, cloning and sequencing of chicken insulin-like growth factor 1 (IGF-1) gene from high producing exotic breed to produce transgenic chickens of native breeds in Egypt

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Abstract

Molecular analysis is an easier means to identify and isolate a specific gene which has imperative function for growth, body composition, fat deposition, metabolic and skeletal traits as well as the molecular genetics selection on individual genes is a very efficient method to genetically improve economically important traits in chickens. Insulin-like growth factor 1 (IGF- 1) is a member of a heterogeneous group of peptides with important growth-promoting effects in vitro as well as *in vivo*. It plays a fundamental role in postnatal chicken growth as a major mediator through which growth hormone exerts its biological effects. In this study, total RNA was isolated from (Cobb 500 breed) liver samples at 50 weeks age from national poultry company in Egypt. cDNA was produced by reverse transcriptase-polymerase chain reaction (RT-PCR). Standard PCR was carried on using two flanking primers containing *HindIII* and *EcoRI* restriction enzymes to generate the whole length of IGF-1 gene. A PCR product of 490bp, including the open reading frame of IGF-1 gene corresponding to the expected theoretical product size, was successfully amplified and double digested with *HindIII* and *EcoR1* and cloned into same Restriction enzymes digested PCR3.1 mammalian expression vector. Interestingly, the cloned sequence was confirmed by DNA sequencing which reveals that, it is cloned in frame position and 99% matching except one nucleotide due to the heterozygous of isolated IGF-1 cDNA. In the present study we report the isolation, molecular cloning and sequencing of full-length IGF-1 cDNA from chicken liver, encoding the IGF-1 protein, can be used as a candidate gene in transgenic Egyptian native breed production based on the IGF-1 protein of exotic high producing chicken population and may give a tool in continuous improvements of the chicken industry concerning a native breed in Egypt.

Keywords: cIGF-1 gene, transgenic chicken, DNA cloning, sequencing, RT-PCR, cobb chickens

Introduction

The intensive application of selection methods in poultry farming has resulted in an increased growth rate and carcass yield (Nicholson 1998), to improve production traits and health simultaneously. It is appropriate to use molecular markers associated with one or two characteristics and used to produce a transgenic chicken carrying this marker in native breed.

Insulin-like growth factors (IGFs) are a group of structurally-related polypeptides that regulate the growth of many types of mammalian cells (Malak *et al.*, 2008). In vivo, Insulin-like growth factors are involved in several biological processes, such as growth, development and metabolism (Stewart and Rotwein, 1996). The insulin-like growth factor gene (IGF1) is a candidate gene for growth, body composition and metabolism, skeletal characteristics and growth of adipose tissue and fat deposition in chickens (Zhou *et al.*, 2005). Hormones such as the growth hormone, IGF, thyroid hormones and insulin, play important and diverse roles in animal growth (Zhou *et al.*, 2005). Most of the functions of the growth hormone in chickens are mediated by insulin-like growth factors (IGF) (Zhou *et al.*, 2005) which stimulate amino acid uptake, glucose metabolism, DNA synthesis (McMurtry, 1998), protein synthesis, and the proliferation of different cell types, and is also involved in the regulation of growth (McMurtry *et al.*, 1997). Growth rate stimulation by IGF is known in many species of animals. Studies have found that there is no direct dependence between the levels of growth hormone (GH) and the growth rate in chickens, and therefore it might be useful to study insulin-like growth factors (Beccavin *et al.*, 2001), as molecular markers for meat or egg production enhancement in native breed.

The objective of this study was to isolate and molecular cloning of cIGF-1, as an important gene from high producing chicken breed to use in production of transgenic chickens of native breed of Egypt.

Materials and methods

Chicken liver samples

Chicken fresh liver samples were immediately excised from Cobb 500 breed at 50 weeks age from national poultry company in Menoufia Governorate, Egypt. The collected liver tissue was rapidly dissected into small pieces using sterile scalpel, immediately stored at -80ºC until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the liver sample using the RNA easy Mini Kit (Qiagen, Austin, TX, USA) following manufacturer's recommendations. RNA was quantified using nano Drop technology with the Epoch Multi-Volume Spectrophotometer System (Biotech, Winooski, VT, USA). 1 µg of RNA was reverse transcribed into first strand cDNA using a SuperScript® II Reverse Transcriptase (Invitrogen, USA) as per manufacturer's recommendations.

Amplification of cIGF-1 cDNA by polymerase chain reaction (PCR)

Chicken IGF-1 cDNA was amplified using Go Taq Green PCR Master Mix (Promega) whereas, PCR reaction was carried out in a 25 µl in an eppendorf tube with 7.5 µL of the master mix , 6 µL of cDNA (50-fold dilution), and 500 nM of each primer. Gene-specific primers were used to amplify a fragment of 490 bp for IGF-1(F: 5-TT*AAGCTT*GCAATGGAAAAAATCAAC-3; R: 5 TT*GAATT*CGGGCATGATCTTACATTC-3), Flanking by *HindIII* in forward primer as underline and EcoR1 in the reverse primer as underline. Thermal cycling conditions for IGF-1, were as following: initial activation of 4 min at 95 °C, followed by 35 cycles of 30 s at 95 °C; 30 s at 55; 30 s at 72 °C, and a final extension of 10 min at 72^0 C. The amplified PCR products were separated by electrophoresis on 1.5% agarose gel in 1X TAE, and stained with ethidium bromide and visualized with UV light of Gel Documentation System. The agarose gel slice containing the relevant cIGF-1 cDNA fragment was excised and purified by gel extraction kit (QIAGEN) according to the manufacturer's recommendation. Colony PCR was done as the same condition as standard PCR except using the transformed *E.Coli* bacterial colony as a DNA template with prolonged initial denaturation temperature to 10 minutes.

Cloning of cIGF-1

PCR product and pCR3.1 vector was double digested by *HindIII* and *EcoR1* (Biolabs, USA) as follow; 1µg of PCR product or the vector were incubated with 4 µl of each restriction enzyme and 3 µl of buffers and dd water up to 30 µl at 37^0 C for 3 hrs (Fig. 1) Then alkaline dephosphorylation of the vector using calf intestinal alkaline phosphatase (CIP) from (Biolabs, USA) for 1 hour. Purification of the reaction was carried out by PCR isolation Miniprep kit (QIAGEN) as manufacture's procedures. DNA ligation was done in 25 µL in an eppendorf tube containing the following mixture (use ice box), (5 µl digested pCR3.1 vector, 5µl DNA fragment, 3.5 µldd water, 5 µl solution I (ligation mixture) then incubate at 16°C overnight or at least 1 h at room temperature. Followed by Competent *E.Coli* transformation by heat shock method, using 5 ul from legated DNA to transform One Shot® TOP10 Chemically Competent *E.Coli* (Invitrogen, USA) by heat shock method, keep on ice for 30 min and 45 sec on 42° C and then on ice 2 min and add 1 ml SOC media then shacking for 1 hour at 37° C /250 rpm.. The transformed cells were grown on LB Medium for overnight followed by plasmid extraction using Miniprep plasmid purification kit (QIAGEN).

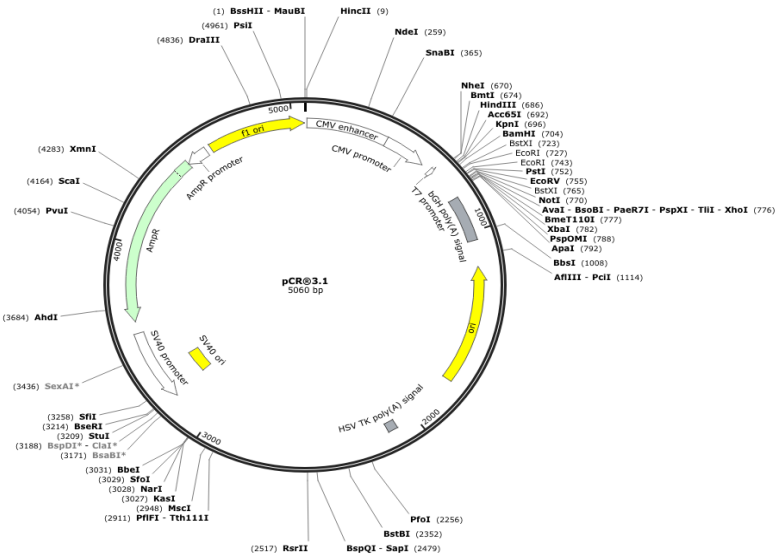

Fig. 1: pCR3.1 vector using to clone the cIGF-1 between *HindIII* and *EcoR1* restriction sites.

Sequencing of cIGF-1 fragment and sequence analysis

Finally, the recombinant plasmid (pCR3.1-cIGF-1) was sequenced using T7 common sequencing primer (TAA-TAC-GAC-TCA-CTA-TAG-GG) by

the Sanger sequencing method at DNA sequencing core (University of Michigan, USA, http://seqcore.brcf.med.umich.edu) and amino acid sequences translated from the cDNA sequence were compared with sequences in the Gen Bank public database, by using (Bioedit software v7.1)

Results

Total RNA was extracted from chicken liver tissue and the cDNA was successfully prepared. PCR amplification with cIGF-1 specific primers generated 490 bp fragment (Fig 2), which was cloned with PCR3.1 mammalian expression vector by double digestion with *HindIII* and *EcoR1* restriction enzymes and relegated with DNA ligase. Chemical competent cells of *E.Coli* were transformed with cIGF-1 recombinant plasmid (Fig. 3) which shows the positive legated clones of cIGF-1 by colony PCR. Plasmid purification and perform standard PCR shows a fragment of about 490 bp when using specific cIGF-1 primers and the same fragment size was generated by double digestion of pCR3.1-cIGF-1 recombinant plasmid (Fig.4). Finally, the recombinant plasmid (pCR3.1-cIGF-1) was sequenced by the Sanger sequencing method at DNA sequencing core (University of Michigan, USA, http://seqcore.brcf.med.umich.edu) to confirm the recombinant cDNA with the Gen Bank reference sequence (accession number NM_001004384.2) (Fig.5,6). The nucleotide and deduced amino acids were aligned and compared with reference sequence which showed about 99% matching due to heterozygous of the extracted cDNA (Fig. 6). Finally the vector map was depicted containing the insert gene by using plasMapper online software (Fig. 7). The result of this study is suggested an easy method to isolate and cloning of a targeted varieties of chicken genes which may be useful to improve the local breed.

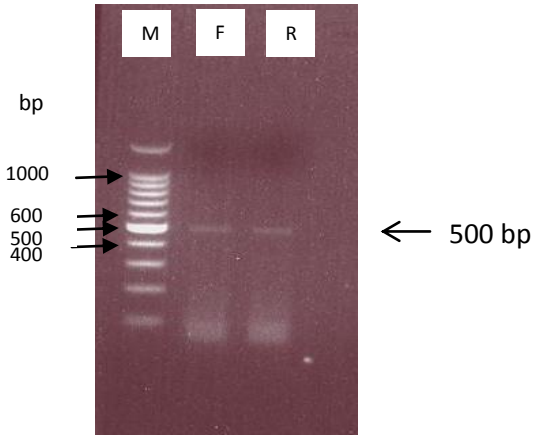

Fig. 2: Agarose gel electrophoresis (1.5%) of PCR products using IGF-1 forward (F) and IGF-1 reverse (R) primers using cDNA as a template. M: 100 bp DNA Ladder (promega).

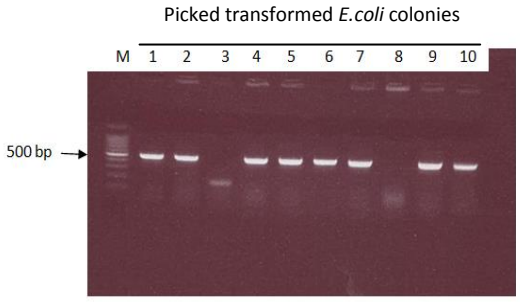

Fig. 3: Agarose gel electrophoresis (1.5%) of colony PCR showing positive colonies form picked transformed *E.coli* colonies (8 of 10 samples) using IGF-1 forward and IGF-1 reverse primers. M: 100 bp DNA Ladder (promega).

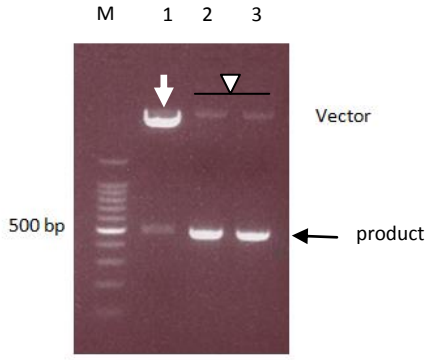

Fig 4: Agarose gel electrophoresis (1.5%) Showing PCR products using IGF-1 (F,R) primers and T7 vector primer with IGF-1 reverse primer to confirm the ligation in frame direction (2,3), while 1 is a digested plasmid with *HindIII* and *EcoR1*which generate the same PCR product. M: 100 bp DNA Ladder (promega). the arrow shows the vector without the insert (digested by the restriction enzymes), while the arrow head refers to the vector containing the insert.

ATGGAAAAAATCAACAGTCTTTCAACACAATTAGTTAAGTGCTGCTTTTGTGATTTCTTGAAGGTGAAGATGC
ACACTGTGTCCTACATTCATTTCTTCTACCTTGGCCTGTGTTTGCTTACCTTAACCAGTTCTGCTGCTGCCGGC
CCAGAAACACTGTGTGGTGCTGAGCTGGTTGATGCTCTTCAGTTCGTATGTGGAGACAGAGGCTTCTACTTC
AGTAAGCCTACAGGGTATGGATCCAGCAGTAGNCGCTTACACCACAAGGGAATAGTGGATGAGTGCTGCTT
CCAGAGTTGTGACCTGAGGAGGCTGGAGATGTACTGTGCTCCAATAAAGCCACCTAAATCTGCACGCTCTGT
ACGTGCTCAGCGCCACACTGATATGCCAAAAGCACAAAAGGAAGTGCATTTGAAGAATACAAGTAGAGGGA
ACACAGGAAACAGAAACTACAGAATGTAA

Fig 5: Sequencing result of cIGF-1 recombinant gene by standard Sanger sequencing method using T7 forward primer.

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                    250       260       270       280       290       300
cIGF-1(NM_001004384.2)  247 atggaaaaaatcaacagtctttcaacacaattagttaagtgctgcttttgtgatttcttgaag
                              M  E  K  I  N  S  L  S  T  Q  L  V  K  C  C  F  C  D  F  L  K
cIGF-1(cloned sequence) 247 ............................................................
                              M  E  K  I  N  S  L  S  T  Q  L  V  K  C  C  F  C  D  F  L  K

                    350       360       370       380       390       400
cIGF-1(NM_001004384.2)  347 accttggcctgtgtttgcttaccttaaccagttctgctgctgccggcccagaaacactgtgtg
                              Y  L  G  L  C  L  L  T  L  T  S  S  A  A  A  G  P  E  T  L  C
cIGF-1(cloned sequence) 347 ............................................................
                              Y  L  G  L  C  L  L  T  L  T  S  S  A  A  A  G  P  E  T  L  C

                    450       460       470       480       490       500
cIGF-1(NM_001004384.2)  447 agacagaggcttctacttcagtaagcctacagggtatggatccagcagtagacgcttacacca
                              D  R  G  F  Y  F  S  K  P  T  G  Y  G  S  S  S  R  R  L  H  H
cIGF-1(cloned sequence) 447 ...........................................N................
                              D  R  G  F  Y  F  S  K  P  T  G  Y  G  S  S  S  X  R  L  H  H

                    550       560       570       580       590       600
cIGF-1(NM_001004384.2)  547 gacctgaggaggctggagatgtactgtgctccaataaagccacctaaatctgcacgctctgta
                              D  L  R  R  L  E  M  Y  C  A  P  I  K  P  P  K  S  A  R  S  V
cIGF-1(cloned sequence) 547 ............................................................
                              D  L  R  R  L  E  M  Y  C  A  P  I  K  P  P  K  S  A  R  S  V

                    650       660       670       680       690       700
cIGF-1(NM_001004384.2)  647 aggaagtgcatttgaagaatacaagtagagggaacacaggaaacagaaactacagaatgtaa
                              K  E  V  H  L  K  N  T  S  R  G  N  T  G  N  R  N  Y  R  M  *
cIGF-1(cloned sequence) 647 ............................................................
                              K  E  V  H  L  K  N  T  S  R  G  N  T  G  N  R  N  Y  R  M  *
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Fig 6: Nucleotide and deducted amino acid sequences alignment generated with Bio Edit Sequence Alignment Software (v7.1).

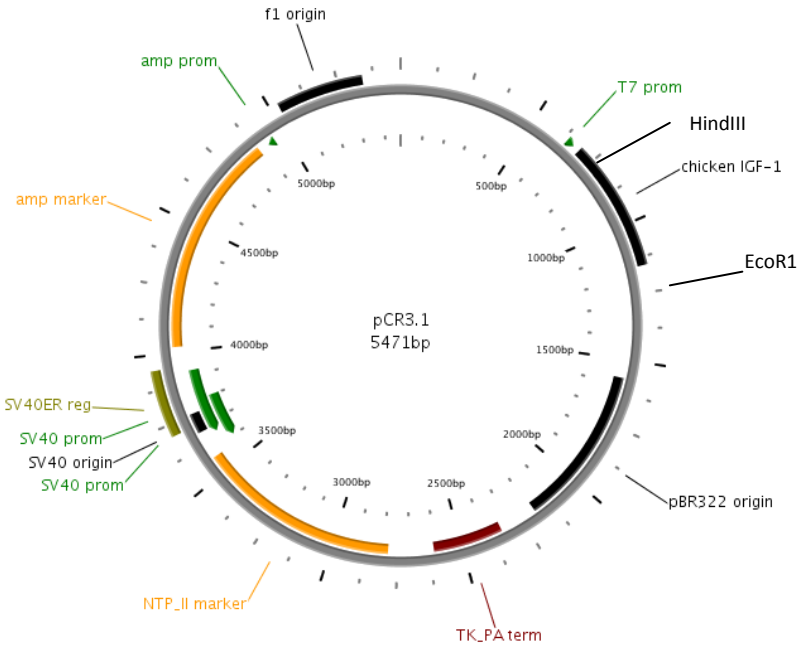

Fig. 7: pCR3.1 vector containing the cIGF-1 gene sequence between *HindIII* and *EcoR1* restriction sites.

Discussion

Molecular biologists exploit the replicative ability of cultured cells to clone genes. Gene cloning also enables scientists to manipulate and study genes in isolation from the organism they came from. This allows researchers to conduct many experiments that would be impossible without cloned genes.

Recombinant DNA technology is another major DNA-based tool that has gained popular attention in the past decade. This technology allows scientists to find individual genes, cut them out, and insert them into the genome of another organism. Recombinant DNA technology has applications in health and nutrition. In medicine, it is used to create pharmaceutical products such as human insulin (Dyck *et al.*, 2003). In agriculture, it is used to impart favorable characteristics to plants for increasing their yield and improve nutritional content Recombinant DNA technology requires the use of molecular scissors called restriction enzymes, which cut DNA at specific sequences. The cut-out gene is then inserted into a circular piece of bacterial DNA called a plasmid. The plasmid is then re-introduced into a bacterial cell. When the bacteria multiply, the plasmids multiply as well, creating many copies of the gene. Since bacteria multiply very quickly, large numbers of the gene can be produced in the laboratory for further analysis and application (Anathy *et al.*, 2001. and Dyck *et al*., 2003).on other hand the transgenic technologies have brought a new aspect into in vivo observation of gene function. It is now possible to insert new functional genes, modify and inactivate the existing ones in living organisms to take the benefit of those genes from one breed to another (Doosti *et al.*, 2013).

From the previous information it could be concluded that a growth candidate gene in broiler chicken can take the advantages of this gene and make a transgenic chicken carrying this promising candidate by the molecular and transgenic tools in local Egyptian chicken breed. Interestingly, IGF-I plays a key role in the development of muscle tissue and positively affects the growth of muscle (Duclos ,2005; Kadlec *et al*., 2011and Escobar et al., 2011), also regulates glucose, fat and muscle protein metabolism (Yun *et a*l.2005). Breast muscle weight and percentage are the most economically valuable traits for broilers. Contrary to the results of Zhou *et al*. (2005), The IGF1 gene is essential for normal embryonic and postnatal growth in mammals (Bian *et al.*, 2008).

From the previous work we can use the power of PCR technique in order to produce a chicken IGF-1 cDNA from extracted RNA .The 490 sequence products was cloned into pCR3.1 mammalian expression vector. The recombinant plasmid was confirmed by sequencing and determined had an open reading frame encoding 153 amino acids with sequence similarity with reference Gen bank sequence.

In a conclusion chicken IGF-1 cDNA successfully isolated from high producing exotic chicken liver tissues and subsequently confirmed the identity of the PCR product by DNA sequencing. this study was easy way to isolate and clone an important gene related to the growth in chicken and so fare can be used as a new tool to produce high phenotypic traits in local breed.

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