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Research Article

POLYMORPHISM AND EXPRESSION OF SOME MYOGENIC GENES AT EMBRYONIC STAGES AND 37 DAYS AGE OF COBB BROILER CHICKENS AND THEIR IMPACT ON THE MARKETING WEIGHTS

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ABSTRACT

Background: *Myostatin* gene (*MSTN*) and myogenic regulatory factors (*MRFs*) have an important role in muscle development. The aim of this study is investigate the expression level of *MSTN* gene and myogenic regulatory factors (*MyoD* and *MyoG*) at embryonic days 7, 13, and 16 and 3days post hatch in Cobb broiler chickens as well as the difference in their expression at the marketing age in higher and lower body weight birds. Also, to screen nucleotide polymorphism in a whole translated region of *MSTN* gene among the two different weights.

Methods: Quantitative real-time PCR was used for assessment of the target genes expression at different embryonic stages of Cobb broiler and at marketing age. Cobb *MSTN* exons were sequenced for their characterization.

Results: The expression profile of *MSTN* and *MyoG* at different embryonic stages showed the highest significant increase at day 7 (125±0.06, 25±0.99 fold), and their expression decrease at E13, 16 and 3 days post hatch. The relative expression level of *MyoD* was significantly high ($p \leq 0.05$) at E7 (42.79±2.03 fold) and reach its peak at E16 (54.95±2.92 fold) and decreased to 6.80±1.30 fold at three days post hatch. At marketing age, there were differences in expression level of *MSTN*, *MyoD*, and *MyoG* in Cobb broiler among high and low body weight birds. Concerning *MSTN* gene sequence data; high similarity in the sequences was detected among high and low body weights at the three exons of Cobb broiler.

Conclusions: There is strong putative role for *MSTN*, *MyoD*, and *MyoG* gene expression at embryonic day 7 in Cobb broiler and this role were also found for these genes at the marketing age that influence bird's weights. Additionally, *MyoD* gene expressed at early and late embryonic life, 3 days post hatch and at marketing age, suggesting its role in muscle differentiation and development.

INTRODUCTION

Skeletal muscle development follows a defined pattern of a temporal gene expression during embryonic and postnatal growth (Bentzinger *et al*., 2012). This process is very similar in mammals, poultry, and fish but with some species-specific differences. Muscle fibers are the functional units of muscle, and their number and size determine muscle mass. In most species, the majority of muscle fibers set at birth (Rehfeldt *et al*., 2011). In chickens, a total number of skeletal muscle fibers remain unchanged post-hatch and its growth is mainly due to hypertrophy (Smith 1963 and Braun and Gautel 2011).

The development of skeletal muscles in an embryo controlled by the myogenic regulatory factors (*MRFs*) including Myogenic factor D1 (*MyoD1*), *Myogenin* (*MyoG*), Myogenic factor 5 (*Myf5*), and Myogenic regulatory factor 4 (*MRF4*) (Berkes and Tapscott 2005). These proteins are belonging to the family basic-helix-loop-helix transcription of the myogenic cell lineage and differentiation of myoblasts in all muscle forming regions of the embryo (Massari and Mure 2000). The primary MRF genes (*MyoD* and *Myf5*) are required for commitment to the proliferation of somatic cells to the myogenic lineage, while, the secondary MRF genes (*MyoG* and *Myf6*) are required for the committed cells to differentiate further into myocytes and from mature into myofibers (Zhang *et al*., 1995).

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The *myoD1* with *Myf5* are mainly expressed in the myoblast proliferation of skeletal muscle cells and subjected to distinct cell cycles regulation. Also, they can independently initiate the myogenic program and thus act as myogenic determination genes. They play redundant roles in the specification and maintenance of myoblasts (Sun 2008). The *MyoG* gene expressed after *Myf5* and *MyoD* and required for the myoblast differentiation established by the initial expression of *Myf5 orMyoD1*genes (Bergstrom and Tapscott 2001).

Myostatin (*MSTN*) is a one member of transforming growth factor-beta super family. It is an essential factor for the growth and development of muscle mass and highly conserved in gene structure among vertebrate species (McPherron and Lee 1997 and McCroskery *et al*., 2003). The *MSTN* regulates growth negatively by limiting chicken muscular growth during the pre-hatch and post-hatch period (Sato *et al*., 2006). During the embryonic stage, *MSTN* reduced the *Pax-3* gene expression, which associated with a proliferation of myogenic cells and prevents expression of *Myo D*gene involved in an activation of the myogenic program (Amthor *et al*., 2002). *MSTN* inhibits the myoblast proliferation (Bass *et al*., 1999) by preventing the progression of myoblasts from G1 to S phase of the cell cycle (Thomas *et al*., 2000). Postnatal, it affects skeletal muscle growth by maintaining the quiescent satellite cells (McCroskery *et al*., 2003).

Studying DNA polymorphisms and causative genes affecting weight provides necessary molecular information for marker-assisted and gene-based selection (Goddard and Hayes 2009). Myostatin is a negative muscle regulator; several studies have associated its nucleotide polymorphism in some exons and intron or promoter with production and carcass traits in chicken (Zhiliang *et al*., 2004; Zhu *et al* 2007 and Paswan *et al*., 2014).

The aims of this study are investigate the expression level of *MSTN* gene and myogenic regulatory factors (*MyoD* and *MyoG*) at embryonic days 7, 13, and 16 and 3 days post hatch in Cobb broiler chickens as well as the difference in their expression at the marketing age in higher and lower body weights birds. Also, to screen nucleotide polymorphism in whole translated region of *MSTN* gene among the two different weights.

MATERIALS AND METHODS

Experimental samples

Sixty fertilized egg were obtained from Tabark lab, Gamasah, Dakahlia governorate, Egypt. They were incubated under proper condition in humidified atmosphere (60-70%) at 37.5 ºC. Embryo samples were collected at different stages, embryonic day 7 (E7), E13, E16 and 3 days post hatch (n=5 per day). At E7 and E13, the whole embryos were collected. While, at E16, 3 days post hatch chicks and at the marketing age (37 days), breast muscles were collected and all samples kept at -80°C for gene expression study.

For studying *MSTN* gene polymorphism, two chicken groups were used: group I, high body weight (1585-2465 gm) and group II with lower weights (0.835-1485 gm). The chickens were obtained from poultry farm at Edfina, Behera governorate, Egypt. These chickens were kept in the same condition, diet and management from the day of brooding till marketing. Blood samples from 32 broiler chickens were collected from wing vein into tubes containingEDTA. The blood samples were kept at -20 C until DNA extraction.

Total RNA Purification, CDNA Synthesis and DNA Extraction

The total RNA was extracted and from a whole embryo and muscle tissue using Biozol (Bioflux, Japan) and the extracted RNA was stored at -80 ºC. The quality of RNA was checked on 2% agarose. cDNA synthesis was performed by SensiFAST™ cDNA Synthesis Kit (Bioline, United Kingdom) according to manufacturer instruction protocol and the obtained cDNA stored at -20°C until further use. Genomic DNA was extracted from blood using Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo, Lithuania) according to manufacturer instruction and the extracted DNA stored at -20 ºC. The quality and integrity of DNA was checked on 2% agarose.

Real time PCR Quantification of Target Genes and Data Analysis

Quantification of target selected genes (*MSTN*, *MyoD* and *MyoG*) was done using Stratagene MX3000P (Clean lab, USA). The RT-PCR reaction was carried out in a total volume of 20µl, consisting of 10 µl of Syber green master mix (Bioline, United kingdom), 0.8 µl of each primer (50nm), 2 µl cDNA (Bioline, United kingdom), 6.4 µl of RNase free water and the program was carried out by an initial heating at 95°C for 10 min followed by 40 cycles of 95°C for 15 second and annealing temperature for 1 min, the primers sequence of the target genes were shown in table (1). The dissociation curve was generated at the end of the last cycle by collecting the fluorescence data at 60°C and taking measurements every 7 sec until the temperature reached 95°C. The quantification analysis was done by comparative threshold cycle (CT) (Livak and Schmittgen 2001). The results were reported as fold change as compared to the calibrator after normalization of the transcript amount with *GAPDH* gene as an endogenous control.

PCR Amplification of MSTN gene, DNA Sequensing and Data Analysis

Amplification of *MSTN* gene exon 1, 2 and 3 fragments which represent whole translated region were performed in 50 µl reaction volume containing 4 µl genomic DNA, 5 µl 10X buffer, 1 µl dNTPs mix 2.5mM/10mm, (Thermo scientific, Lithuania), 1 µl of each primer (50 nm) (Invitrogen, USA)**,** 0.6 µl of Taq DNA polymerase 50 ul, 500U (Thermo scientific, Lithuania) and 37.4 µl dH_2O which finally added. The final reaction mixture was placed in thermal cycler (Technee, TC-3000, USA) and the PCR program was carried out by initial denaturation at 95 ºC for 5 min followed by 35 cycles of 94 ºC for 1 min for DNA denaturation, annealing temperature was 55 ºC (Bhattacharya and Chatterjee 2013) for 1 min, extension at 72 ºC for 1 min and final extension at 72 ºC for10 min. The primers sequences of the amplified exons were shown in table (1).

Table 1 List of primers used in this study

Primers	Sequence (5`→3`)	Annealing temperature (°C)	Amplicon size (bp)
Myostatin	F: GCAAAAGCTAGCAGTCTATG R: TCCGTCTTTTTCAGCGTTCT	59	119
Myo D	F:GATTTCCACAGACAACTCCACAT R: GAATCTGGGCTCCACTGTCACT	60	116
Myo G	F: GTGGGATGGTGATGCTGGAA R: TTGGAGAGGAGTGGGAAAGGA	60	109
GAPDH	F: AACCCATTTTTAGAGGTCAGAG R: TCATGAGCACCCGCAACGAT	60	147
MSTN (Exon 1)	F: ATGCAAAAGCTAGCAGTCTATG R: ACTCCGTAGGCATTGTGATAAT	55	373
MSTN (Exon 2)	F:CTGATTTTCTTGTACAAATGGAG R: CAATCCATCTTCACCCGGTC	55	374
MSTN (Exon 3)	F: AACCCATTTTTAGAGGTCAGAG R: TCATGAGCACCCGCAACGAT	55	381

The PCR products of *MSTN* gene were purified using EZ-10 spin column PCR product purification kit (Bio Basic INC, Canada) according to manufacturer instruction, the purified PCR products were sequenced using forward primers in ABI 3730XL DNA sequencer (Applied Biosystem, USA).

The sequence results were analyzed using Chromas 1.45 (http: //www.technelysium.com.au). Sequence comparisons were performed using the BLAST program from National Center of Biotechnology information website http:// www.ncbi.nlm.nih.gov /. The comparison was done with gb|GU181322.1, gb|GU181325.1 and gb|GU181326.1 for exon 1, 2, and 3 respectively. The alignment of obtained sequences done by Clustalw version 1.8 (Thempson *et al*., 1994) and the amino acid translation was done using MEGA 6. The whole translated region of Cobb broiler was submitted to the genebank and receive the accession No KY203797 and was compared with Gallus gallus strain PB-1 breed Broiler *MSTN* mRNA, complete cds) accessionNo.GU075927.1.

RESULTS

Expression levels of selected genes

In the present study, the expression level of *MSTN* gene in different embryonic stages was the highest significant (P≤ 0.05) at embryonic day 7 (122.86±0.06 fold) (Fig. 1). Also, the relative expression level of *MyoD* was significantly high (P≤ 0.05) at E7 (42.79±2.03 fold) and reach its peak at E16 (54.95±2.92fold) while the expression of *MyoG* showed the highest significant (P≤ 0.05) expression of E7 (22.02±0.99 fold) and its expression decreased from E13 and E16 (Fig. 1).

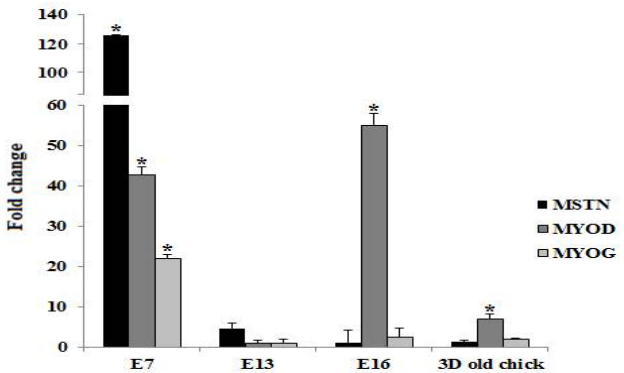

Fig 1 Expression level of selected genes in different embryonic stages E7, E13, E16 and 3 days old chick

Three days post-hatch, mRNA transcripts of *MSTN* and *MyoG* genes are very low, however, *MyoD* transcript level is lower than the embryonic stage, but it is significantly higher (6.80±1.30 fold) than *MSTN* and *MyoG* (Fig. 1).

Considering the expression of selected genes *MSTN*, *MyoD* and *MyoG* in Cobb broiler at marketing age (37 days), the result showed that, the expression level of *MSTN* was significant higher in low body weight relative to high body weight. In contrast, the *MyoD* and *MyoG* expression were higher in high body weight relative to low body weight (Fig. 2).

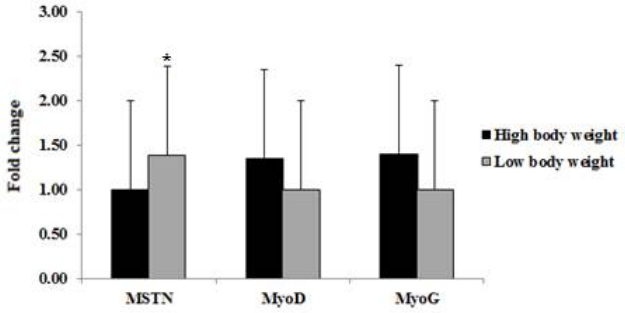

Fig 2 Expression level of selected genes in high and low body weight Cobb broiler at 37 days of age

Myostatin gene polymorphism

The PCR products of exon 1 (373 bp), exon 2 (374bp) and exon 3 (381bp) from the studied birds of Cobb broiler were illustrated in figure 3a and b.

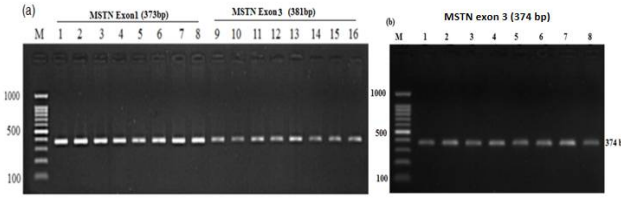

Fig 3 PCR products of *MSTN* exons.(a) Showed exon 1 (373bp) and exon 3 (381bp) from high body weight (1-4) and low body weight (5-8) of exon 1 and (9-12) high body weight and (13-16) low body weight for exon 3 of Cobb broiler chickens. (b) Showed exon 2 (374bp) from high body weight (1-4) and (5-8) low body weight of Cobb broiler chickens.

DNA sequence alignment of exon1,2 and 3 of *MSTN* gene among high and low body weight Cobb broiler showing 100% similarity (Fig. 4). The comparison of Cobb *MSTN* with gene

bank (Gallus gallus strain PB-1 breed Broiler myostatin (MSTN) mRNA, complete cds) accessions No. GU075927.1, revealed six SNPs (51 A>G), (195 C>G), (324 T>C) in exon 1, (408 G>A) in exon2, (966 G>T) and (1120 G>T) in exon 3. The translated amino acids of studied coding region of Cobb *MSTN* gene were it consists of 375amino acids (Fig.4).

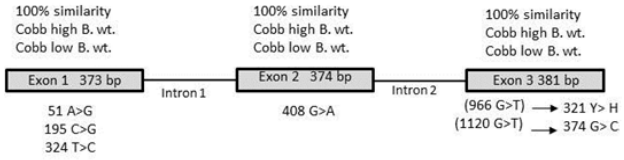

Fig. 4 Cobb broiler *Myostatin* gene structure showing the amplified exons (gray color). The upper part shows the similarity percentage among four sequences for high and low body weights of Cobb broiler at the three exons. Lower parts show the detected SNPs at the three exons compared with gb|GU181322.1, gb|GU181325.1 and gb|GU181326.1 for exon 1, 2, and 3 respectively.

The amino acid alignment of *MSTN* gene of Cobb broilers as compared to gene bank accession number ACY68210.1showed in figure (5). The Two non-synonymous SNPs were detected in exon 3 lead to change in the amino acid where 321 Tyrosine (Y)> Histidin (H) and 374 Glycine (G)> Cysteine (C).

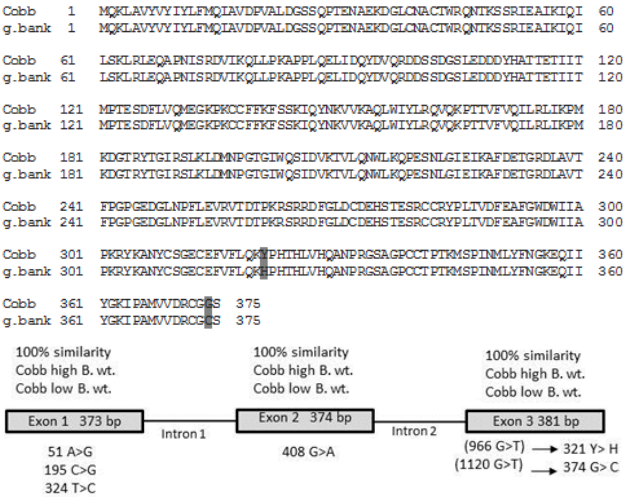

Fig 5 Amino acid alignment of *Myostatin* gene of Cobb broiler compared with broiler strain ACY68210.1.Two amino acid changes marked with gray color: 321 Tyrosine (Y)>Histidin (H) and 374 Glycine (G)> Cysteine (C).

DISCUSSION

Cobb broiler are the one of most effective broiler worldwide, has the best growth rate and lowest cost per kilogram or pound of live-weight (FAO 2013). Breast muscle is a valuable meat product of chicken, however, embryonic and fetal development are characterized by rapid growth and cell differentiation that affect later life (Miller and Stockdale 1986).

In this study, we investigate the expression level of myostatin and myogenic factors in different embryonic stages of Cobb broiler and the result revealed that the expression level of *MSTN* gene was significantly high at E7 relative to E16 (Fig. 1). Ban *et al*., (2013) studied expression level of *MSTN* gene in chicken and quail embryo from 7-17days and they found that its expression level reaches its first peak at E7 in quail and E9 in chicken and this coincide with the period of primary and secondary fiber formation in chicken embryo and the difference between chicken and quail may be due to species difference. Down regulation of *MSTN* gene in our study at the embryonic days 13, 16 and three days post hatch were also reported by Gu *et al*., (2013) in Pekin duck embryos, they studied its expression at embryonic days 11, 14, 17, 20, 23, and 26, they observed decline in its expression to reach its minimum level at E19 and then increase gradually to E26.

Regarding the expression of *MSTN* gene in broiler chickenat marketing age (37 days). The expression of *MSTN* was higher in low body weight relative to high body weight groups. Bhattacharya *et al*., (2014) found thatthe lowest expressions of *MSTN* in broiler (CB- strain and PB_1 line) and layer (IWI line) were found at 6 weeks of age which allow muscle growth and get better body weight. On the other hand, no association between its expression and increased body weight was observed in Taihu and Wanxise geese at the marketing age (Tang *et al*., 2013).

In the present study, the expression of *MyoD* gene was prominent at early and late embryonic stages, its transcript increased to 42±2.03 at E7 and increased to 54.59±2.92 at E16, while its expression was not observed at E13. Supporting the role of this gene expression during primary muscle fiber formation which occurs atembryonic days (Ed) 4 and 7 and also secondary muscle fibers development from ED7-15 (Miller and Stockdale 1987) and also in muscle fiber hyperplasia (Al-Musawi *et al*., 2011).

The highest expression of *MyoG* was observed at E7 (25±0.09), and its transcripts are not prominent during the other embryonic days. Bentzinger *et al*., (2012) suggested that *MyoG* is more directly involved in the myoblasts differentiation process and trigger the expression of myotube-specific genes in the myogenesis.

The combined increased expression of *MSTN*, *MyoD* and *MyoG* genes in our study at the E7 supporting their role at early phase of myogenesis. However, combined down-regulation of the three genes was observed at 3 days posthatch compared with their levels at early embryonic stage. Lee *et al*., (2014) observed a low level of *MyoG* and *MyoD* expression from (0-3) old post hatch period compared with their transcripts at embryonic stages in two lines of Japanese quails, and the level of expression was lower in low weight lines compared with random breed control.

On the other hand, the expressions of both *MyoD* and *MyoG* were higher in high body weight group relative to low body weight group (Fig. 2). Similarly, Yin *et al*., (2014) reported that the relative expression level of *MyoG* and *MyoD* in pectorals muscle were greater in high weight selection than low weight selection at 28 days and 56days of adult broiler.

Concerning *MSTN* gene sequence data; high similarity in the sequences were detected among high and low body weights at the three exons of Cobb broiler, Ahad *et al*., (2016) did not detect any polymorphism at *MSTN* coding regions in ten Bakerwal Goats (Capra hircus), furthermore, the detected SNPs at *MSTN* gene and its association with productive traits usually

involves different lines or breeds of chickens (Ye *et al*., 2007). However, Zhang *et al*., (2010) identified only variant synonymous mutation at exon 1 in *MSTN* coding region which has the potential to be a genetic marker for body weight traits in female Bian chicken.

Comparison of Cobb broiler *MSTN* with Gallus gallus strain PB-1 breed broiler revealed presence of three synonymous SNPs at exon 1, one at exon 2 and two non-synonymous SNPs at exon 3 (966 G>T) and 1120 G>T altering 321Y> H and 374 G> C. Hubbard (2004) suggested that non-synonymous SNPs at highly conserved sites as MSTN gene is likely to affect function (intolerant effect). We suggested that the changes in Cobb *MSTN* protein sequence could modify its growth compared with other broiler because its role is regulation of skeletal muscle growth.

CONCLUSION

In conclusion, there are strong putative role for the expression of *MSTN*, *MyoD* and *MyoG* at embryonic day 7 in Cobb broiler chicks this role also found for these genes at the marketing age that influence bird's weights. Also, *MyoD* gene is expressed at early and late embryonic life, 3 days post hatch and at marketing age, suggesting its major role in muscle differentiation and develop.

Authors' contributions

SAH and AFE conceived and designed the experiments. AAMF and WSHA performed the experiments and AFE and WSHA analyzed the data. AAMF wrote the paper. SAH, AFE and WSHA authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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