Alternative Transcription of Peroxisome Proliferator-Activated Receptor Gamma in the Liver Is Associated with Fatness of Chickens

ABSTRACT

The expression of four transcription variant of peroxisome proliferator-activated receptor gamma gene (PPARG) (XM_015292931.1; XM_015292932.1; XM_015292933.1 and NM_001001460.1) in the liver of broilers was measured and its correlation with abdominal fat weight and relative abdominal fat content was investigated. The study was conducted with 92 slow-growing crossbred chickens (Cobb males x indigenous Green-legged Partridge female chickens) divided into “fat” and “lean” groups, according to their abdominal fat yield. The NM_001001460.1 transcript was upregulated with ratio of means 4.26 (p≤0.01) in the “fat” group in relation to the “lean” group. Expression of this transcript was highly correlated with relative abdominal fat content (0.71, p≤0.01) and abdominal fat weight (0.59, p≤0.01). Two SNPs are located in putative transcription factor binding sites. Mutation -991C>A disrupts PPAR while mutation -884C>T disrupts C/EBP putative binding site. The gene expression analysis of PPARg showed that the expression of the transcripts (NM_001001460.1) was more than four times higher in fat than in lean chickens. These results point out that the peroxisome proliferator-activated receptor gamma NM_001001460.1 transcript could be candidate gene for determination of abdominal fat deposition in the chickens.

INTRODUCTION

Poultry breeding programs are used to improve growth performance and carcass traits. Decades of intensive genetic selection have resulted in higher body weight gain and growth rate, and better feed conversion efficiency in broiler chickens (Wang et al., 2012). However, the selection for rapid growth had unintended effects such as excessive fat deposition. Excessive adiposity is a major problem in meat-type chicken production. About 20% of the broiler’s body weight is fat, and it is mostly deposited in adipose tissues (Havenstein et al., 2003). Fat is currently considered a by-product of very little commercial value. Moreover, it is a body component that requires high energy intake and, therefore, high fat deposition can considerably decrease feed efficiency. The selection of chickens for leanness is one of main directions of poultry breeding (Fouad & El-Senousey, 2014). Abdominal fat weight and abdominal fat percentage are major phenotypic indices of fat traits.

In birds, lipogenesis takes place primarily in the liver, whereas the adipocyte serves as storage site for triglycerides (Cogburn et al., 2004). Hepatic lipogenesis contributes for more than 70% of the fat acids stored in adipose tissue (Richards et al., 2003) because the lipogenic activity is much greater in the liver than in adipose tissue in chickens (O’Hea & Levelle, 1968; Cai et al., 2009). Peroxisome proliferator-activated receptors (PPARs) are the master regulators for the development of adipocytes and lipid metabolism (Royan & Navidshad, 2016). PPARs...
Alternative Transcription of Peroxisome Proliferator-Activated Receptor Gamma in the Liver Is Associated with Fatness of Chickens

MATERIALS AND METHODS

Birds

The experimental procedures were approved by the Ethics Committee of the Polish Academy of Science, under protocol number 27/2009 within the project "BIOFOOD innovative, functional products of animal origin".

This study was conducted with slow-growing chickens derived from a cross between Cobb (C) males and females of an indigenous Polish chicken breed, the Green-legged Partridge (GP). Cobb broilers present excellent feed conversion ratio, fast growth rate, and the ability to thrive on low-density, low-cost feeds, whereas GP chickens are well adapted to extensive management, are able to graze and are resistant to diseases. Crossbred C x GP chickens are characterized by low body weight, typical for slow-growing chicken, and good muscling, especially of the breast.

For the first two weeks, the chickens were kept indoors under a head radiator and received 24 h of light/day. Afterwards, they were allowed to use free ranges (2 birds/m²) and provided 18 h of light inside the shed. All birds were kept in the same controlled environmental conditions until 63 days of age and received the same diets.

A four-phase feeding system was applied, with the supply of starter (0-2 weeks), grower 1 (3-4 weeks), grower2 (4-5 weeks) and finisher (from 6 weeks) feeds. The starter diet contained 3080 kcal ME/kg and 21.3% CP, the grower 1 diet contained 3160 kcal ME/kg and 20.8% CP, the grower 2 diet contained 3180 kcal ME/kg and 19.9% CP, and the finisher diet contained 3200 kcal ME/kg and 19% CP. Feed and drinking water were offered ad libitum.

At 63 days of age, the chickens were electrically stunned in a water bath (120 mA, 50 Hz) for 2 s, and then slaughtered by severing the cervical blood vessels and bled for ca. 3-4min. Out of the 425 total birds slaughtered, 92 birds were randomly selected and divided into two groups with similar abdominal fat percentage and body weight. The “fat” group, including 48 individuals, was characterized by high abdominal fat content (4.00-6.60%), and the “lean” group, with 44 birds, was characterized by low abdominal fat content (0.88-2.54%). The characteristics of the two groups of birds are presented in Table 1. Liver tissue samples about 100 mg from were collected the left lobes, immediately frozen in liquid nitrogen and then stored at -80°C until further analysis.

### Table 1 – Characteristics of the two groups of birds

<table>
<thead>
<tr>
<th>Trait</th>
<th>“fat group”</th>
<th>“lean group”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live body weight, g (LBW)</td>
<td>2840 ± 215.40</td>
<td>2516 ± 241.55</td>
</tr>
<tr>
<td>Carcass weight, g (CW)</td>
<td>2102 ± 211.55</td>
<td>1862 ± 254.65</td>
</tr>
<tr>
<td>Abdominal fat, g (AF)</td>
<td>89 ± 20.9</td>
<td>41 ± 10.1</td>
</tr>
<tr>
<td>Abdominal fat yield, %</td>
<td>4.2 ± 0.79</td>
<td>2.2 ± 0.43</td>
</tr>
</tbody>
</table>

Isolation of nucleic acids

Genomic DNA was extracted using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions (Sambrook et al., 1989). After purification DNA was eluted in 100 μL H₂O. The amount of DNA and 260/280 ratio were measured with a NanoDrop
Alternative Transcription of Peroxisome Proliferator-Activated Receptor Gamma in the Liver Is Associated with Fatness of Chickens

2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) (Malewski et al., 2010). Extracted DNA was stored at -20°C.

Total RNA was extracted with a TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions (Farrell, 1998). Briefly, about 100 mg of liver tissue were homogenized in 1 mL of TRI Reagent and incubated for 5 minutes at room temperature for complete dissociation of nucleoprotein complexes. After the addition of 0.2 mL chloroform, the samples were vortexed and incubated at room temperature for 15 minutes. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 4°C. The water phase was transferred to a fresh Eppendorf tube and RNA precipitated with isopropanol. The obtained RNA was stored at -80°C. The amount of total RNA extracted was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was electrophoretically checked in 1.5% agarose gel stained with ethidium bromide (Malewski et al., 2015). Only samples with high integrity were used for further analyses.

cDNA synthesis

In order to prevent probe contamination by genomic DNA, the total RNA was treated with RNase free DNase I (Sigma-Aldrich, St. Louis, MO, USA). One µg of RNA was treated with 1U of DNase I for 15 min at room temperature. The reaction was stopped by adding the stop solution, and DNase I was inactivated at 70°C for 10 min. cDNA synthesis was carried out using Enhanced Avian HS RT-PCR Kit (Sigma-Aldrich, St. Louis, MO, USA), according the manufacturer’s instructions. Briefly, 1 µL of anchored oligo (dT)23 was added to 1 µg of DNase-treated RNA and incubated for 10 min at 70°C. After that, all remaining components were added and the reaction was run at 45°C for 50 min. The cDNA was used immediately in the PCR or stored at -20°C (Ciechanowska et al., 2008).

Primer design

Transcript-specific primers were designed using data information from the chicken genome assembly Gallus_gallus-5.0 (GenBank Acc NC_006099.4) using the software PRIMER 3 http://bioinfo.ut.ee/ primer3-0.4.0 (Koressaar & Remm, 2007; Untergasser et al., 2012). The primer sequences are presented in Table 2. GAPDH was used as reference gene as in our previous experiments (Larkina et al., 2011). We applied the following primers for GAPDH expression profiling: GAPDHfw, CCTCTCTGGCAAAGTCCAAG and GAPDHrv, CATCTGCCCATTTGATGTTG. PCR primers were supplied by Sigma-Aldrich (Sigma-Aldrich, Milwaukee, WI, USA). Three pairs of primers were designed for sequencing the 5’-flanking region of NM_001001460 from -1944 to +15 (Table 3).

qPCR

The expression of PPARG transcript variants was measured by qPCR with the primers presented in Table 2. The obtained amplicons had a single melting peak and showed a single band after electrophoresis in 3% agarose gel, which suggests that the primers amplify specific cDNA regions. qPCRs were performed on the RotorGene 6000 system using LuminoCt SYBR Green

### Table 2 – Primers used for gene expression analysis

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sequence</th>
<th>Tm</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XM_015292931.1</td>
<td>FW: TATCCCACCAGAAGGGAAACA</td>
<td>60.31</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>RV: ATGATCATCCATCGCAGACA</td>
<td>60.04</td>
<td></td>
</tr>
<tr>
<td>XM_015292932.1</td>
<td>FW: ACATTCACATTATGGTGCAATCA</td>
<td>58.56</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>RV: TGAAAATCCAGAGGCCTTGTC</td>
<td>60.20</td>
<td></td>
</tr>
<tr>
<td>XM_015292933.1</td>
<td>FW: CGGCCGTCCGGAGGATTACA</td>
<td>58.62</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>RV: TGTTGTTGAAGAATGCTGGAA</td>
<td>59.21</td>
<td></td>
</tr>
<tr>
<td>NM_001001460.1</td>
<td>FW: CAAGCTCAGGATTGCAGAAG</td>
<td>59.80</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>RV: TGAAATCCAgGGGCCTTGTC</td>
<td>60.20</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3 – Primers used for amplification and sequencing of NM_001001460 5’-flanking region

<table>
<thead>
<tr>
<th>Localization of amplified region regarding to transcription start point</th>
<th>Sequence</th>
<th>Tm</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+15 - -760</td>
<td>FW: TAACCTTCTCGAGCCTTGCC</td>
<td>59.59</td>
<td>746</td>
</tr>
<tr>
<td></td>
<td>RV: TGTGGTGGCAAGCCAGATGA</td>
<td>60.24</td>
<td></td>
</tr>
<tr>
<td>-741 - -1408</td>
<td>FW: TGTAAGCGGCTACAGGAGGGA</td>
<td>59.94</td>
<td>668</td>
</tr>
<tr>
<td></td>
<td>RV: GCAGGGCTCGAGGAAAGTTA</td>
<td>59.59</td>
<td></td>
</tr>
<tr>
<td>-1292 - -1944</td>
<td>FW: TGCCGCCCACCAGGCAACAT</td>
<td>59.96</td>
<td>653</td>
</tr>
<tr>
<td></td>
<td>RV: GCCCTTCTTATCAGCTTGT</td>
<td>59.55</td>
<td></td>
</tr>
</tbody>
</table>
Alternative Transcription of Peroxisome Proliferator-Activated Receptor Gamma in the Liver Is Associated with Fatness of Chickens

Expression of PPARG transcript variants

Based on the genomic structure of PPARG given in GenBank NC_006099.4 record, four variant-specific primer pairs (Table 2) were designed. Using these primers, we performed PCR on the cDNA synthesized on total RNA extracted from chicken livers. The obtained amplicons yielded a single product band with the expected size (136, 153, 159 and 159 bp for transcripts XM_015292931.1, XM_015292932.1, XM_015292933.1 and NM_001001460.1) on agarose gel electrophoresis. The amplified PCR products were sequenced and their proving amplification of the of PPARG gene transcript.

The expression of the four transcript variants was investigated by qPCR in the liver of broilers with relative abdominal fat contents of 0.88% to 6.62%. The obtained results showed that all four transcripts were expressed in the liver (Fig. 1). The expression level of all four transcript was similar in the liver of the “lean” group. The expression of three of the four transcript

Amplification and sequencing of 5'-flanking region of NM_001001460.1 PPARG gene transcript

A fragment of the PPARG gene 5'-flanking region (from -1944 to +15 according to transcription start point) was amplified using three pairs of primers (Table 2).

Amplification was performed using reactions containing 20 μL RedTaq Ready Mix (Sigma-Aldrich), 3 μL of the corresponding primer (5 μM), 2 μL DNA template, and H₂O to a total volume of 40 μL. All PCR reactions were performed in Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) as follows: an initial denaturation step at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min with a final incubation for 5 min at 72°C.

Excess dNTPs and unincorporated primers were removed from the PCR product using the Clean-Up Purification Kit (A&A Biotechnology, Gdynia, Poland). As a final step, the purified DNA was eluted in 40 μL H₂O.

Sequencing PCR reactions consisted of 1 μL BigDye Terminator v. 3.1 Ready Reaction Mix (ThermoFisher Scientific, Waltham, MA, USA), 2 μL BigDye sequencing buffer (ThermoFisher Scientific), 1 μL (5 μM) forward or 1.6 μL (10 μM) reverse primer and H₂O to 10 μL total volume. The thermal profile for sequencing reactions consisted of an initial denaturation step at 96°C for 1 min followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 105 s. PPARG gene 5'-flanking region was sequenced with an ABI 3500XL genetic analyser (Applied Biosystems).

In-silico analysis of 5'-upstream sequences

The 5'-flanking regions (2.0 kb) upstream of the transcripts XM_015292931.1; XM_015292932.1; XM_015292933.1 and NM_001001460.1 of the PPARG (GenBank accession no. NC_006099.4) were analysed for putative transcription factor binding sites using the program LASAGNA2 (Lee and Huang, 2013), based on TRANSFAC database matrices for vertebrates. Localization of analyzed 5'-upstream sequences: XM_015292931.1 - 4827225-4829225; XM_015292932.1 - 4827447-4829447; XM_015292933.1 - 4857857-4859857 and NM_001001460.1 - 4858927-4860927. A cut-off p-value of 0.001 was applied. The CpG islands were predicted using the CpG Finder (http://www.softberry.com) program.

RESULTS

Expression of PPARG transcript variants

Based on the genomic structure of PPARG given in GenBank NC_006099.4 record, four variant-specific primer pairs (Table 2) were designed. Using these primers, we performed PCR on the cDNA synthesized on total RNA extracted from chicken livers. The obtained amplicons yielded a single product band with the expected size (136, 153, 159 and 159 bp for transcripts XM_015292931.1, XM_015292932.1, XM_015292933.1 and NM_001001460.1, respectively) on agarose gel electrophoresis. The amplified PCR products were sequenced and their proving amplification of the of PPARG gene transcript.

The expression of the four transcript variants was investigated by qPCR in the liver of broilers with relative abdominal fat contents of 0.88% to 6.62%. The obtained results showed that all four transcripts were expressed in the liver (Fig. 1). The expression level of all four transcript was similar in the liver of the “lean” group. The expression of three of the four transcript
variants was higher in the “fat” group; however, only one (NM_001001460.1) was statistically significant. The NM_001001460.1 transcript was upregulated in the liver with ratio of means of 4.26 (p≤0.01) in the “fat” group relative to the “lean” group. The results showed a highly significant correlation between the expression level of the PPARG transcript variants in the liver and carcass fatness, as shown in Table 4.

**Table 4** – Correlation of the expression of PPARG transcript variants in the liver of chickens

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Ratio of means fat/lean</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>XM_015292931.1</td>
<td>2.16</td>
<td>0.36</td>
</tr>
<tr>
<td>XM_015292932.1</td>
<td>1.63</td>
<td>0.41</td>
</tr>
<tr>
<td>XM_015292933.1</td>
<td>0.96</td>
<td>0.11</td>
</tr>
<tr>
<td>NM_001001460.1</td>
<td>4.26**</td>
<td>0.71**</td>
</tr>
</tbody>
</table>

Figure 1 – Expression of PPARG gene transcripts in “lean” and “fat” broilers.

The expression of this gene was highly correlated with relative abdominal fat content (0.71, p≤0.01) and abdominal fat weight (0.59, p≤0.01). This is the first report on the correlation between transcript variants of PPARG in the liver and fatness in chickens.

**Characterization of PPARG 5’-upstream sequences**

The analysis of four PPARG 5’-upstream flanking regions for putative transcription factor binding sites showed that these sites can be divided into three groups: group I – putative binding sites present in all analyzed 5’-upstream sequences - 32 transcription factors, group II - putative binding sites shared by several 5’-upstream sequences - 59 transcription factors, and group III - putative binding sites unique for 5’-upstream sequence of particular transcript (Table 5). The results showed putative binding sites for several transcription factors important for adipogenesis, lipogenesis and lipolysis: CCAAT/enhancer binding protein (C/EBP), (from -877 to -891 and from -1965 to -1979), hepatocyte nuclear factor 4 (HNH-4) (from -231 to -244; from -1569 to -1582 and from -1682 to -1695), PPAR:RXR (from -983 to -1002, from -1461 to -1475 and from -1685 to -1699), sterol regulatory element binding transcription factor (SREBP) (from -473 to -484). Especially interesting was the presence of three putative binding sites of PPAR:RXR. These transcription factors putative binding sites were found only in the 5’-upstream sequence of NM_001001460.1, which may suggest the existence of a PPARG autoregulatory feedback loop. In addition, PPAR:RXR putative binding sites for other six transcription factors (E4BP4, ER, Gfi-1, HNF-1, MIF-1, PPARalpha:RXR-alpha and RSRFC4) were found at 5’-upstream sequence of NM_001001460.1 only.

CpG island was found in 5’-upstream sequence of XM_015292931.1 transcript in position -1 - -312 bp (73.4% CG) and 5’-upstream sequence of XM_015292932.1 transcript in position -1 - -534 bp.
DISCUSSION

Lipogenesis occurs both in the adipose tissue and liver in mammals, whereas in avian species, the liver is the main lipogenic site. Gene expression analysis shows that the expression patterns of adipogenic transcription factors are different between mammals and avian species both in vitro and in vivo (Matsubara et al., 2005).

Previous analysis showed that PPARG expression is more than 3-fold upregulated in “fat” chickens. The primer pairs used in that analysis, however, measured the expression of all gene transcripts (Larkina et al., 2011). The design of transcript specific primers allowed us to measure the expression of each transcript reported for the PPARG gene (GenelID:373928). The expression of three PPARG gene transcript variants was upregulated in the liver of the “fat” chickens relative to the “lean” chickens; however, only the expression of the NM_001001460.1 transcript was statistically significant (4.26-fold, p ≤ 0.01). Its expression is significantly correlated both with abdominal fat weight (0.59, p ≤ 0.05) and relative abdominal fat content (0.71, p ≤ 0.01) (Table 3). Alternative splicing of pre-mRNA plays an important role in regulating gene expression in higher eukaryotes. Alternative splicing of several genes has been reported to be important in adipogenic pathways: PPARγ (Mueller et al., 2002), Pref-1 (Mei et al., 2002), MC2R (Noon et al., 2006), ACBP (Ludewig et al., 2011). The three alternative splicing isoforms α, δ, and γ of the PPARG gene were detected in laying hens liver, and the isoform γ was significantly down regulated in 30-week-old compared with 20-week-old hens, while isoforms α and δ were not differentially expressed (Li et al., 2015). Differences in the expression of PPARG in chicken were reported by Duan et al. (2015); however, the transcripts described by them differ from transcripts reported in GenelID:373928.

The correlation between PPARG expression and obesity was reported in humans (Hindle et al., 2009). Chen et al. (2009) reported that mutations in ESR1 and PPARG were genetically linked with obesity in Han Chinese. In mammals, PPARG is present in two isoforms, PPARG1 and PPARG2, generated by alternative promoter usage. PPARG mediates the expression of fat-specific genes and activates adipocyte differentiation (Matsubara et al., 2005). The expression of both γ1 and γ2 mRNAs was abundant in mouse adipose tissue (Vidal-Puig et al., 1996). At lower levels, PPARG1 expression was also detected in heart, liver and spleen, while γ1 and γ2 mRNA were found in the skeletal muscle, and fasting reduced PPAR gamma protein levels in adipose tissue. Wang et al. (2008) have shown that the transfection of in-vitro synthesized small-interference PPARG RNA (siPPARG) in cultured preadipocytes of 12-d-old chickens significantly inhibited the differentiation and stimulated the proliferation of preadipocytes. Sato et al. (2004) also reported a correlation between PPARG expression and fat deposition in broilers. Different amounts of PPARG proteins were found in the adipose tissue of divergently selected broilers (Wang et al., 2009). Li et al. (2005) showed that PPARG affects chicken fat metabolism and that SNPs can be used in molecular assistant selection as a genetic marker for the chicken fatness traits. Our investigations of PPARG transcript variant expression in the liver of chickens showed that the expression of only one transcript variant is correlated with carcass fat content (Table 4).

Despite the importance of PPARG expression for adipogenesis and lipid metabolism, there are few data on its gene promoter analysis. Deletion analysis of NM_001001460.1 transcript 5'-upstream sequence ofXM_015292933.1 and NM_001001460.1 Resequencing of 5'-flanking region of NM_001001460.1 showed the presence of four SNPs: G>A at position -353, C>T at -884, C>A at -991 and A>C at 1348. Two of them change putative transcription factor binding site: mutation -991C>A disrupts PPAR, while mutation -884C>T disrupts C/EBP putative binding site. Two other mutations, -353G>A and -1348A>C, did not affect transcription factors binding sites.
C/EBP binding sites were found in all transcripts, while ATF, HNF and SREBP only insome transcripts of the 5'-upstream sequence (Table 5). The presence of three putative PPAR binding sites at 5'-upstream region of NM_001001460.1 transcript suggests that one activated transcription of this transcript variant can be maintained throughout the chicken's lifespan. Unfortunately, there is no information about other transcription factors (E4BP4; ER; Gfi-1; HEN1; MIF-1; PPAR, RSRFC4) specific for this transcript 5'-upstream sequence in the chicken liver (Table 5). In chickens, Hu et al. (2010) reported an association of SNP in PPARG promoter with abdominal fat weight. A significant effect of PPAR gamma polymorphisms on the intramuscular fat content of the longissimus dorsi muscle was reported for Erhualian pigs (Wang et al., 2013).

**CONCLUSION**

Excessive adiposity has become a major drawback in meat-type chicken production, and fat is currently considered a by-product of very little commercial value. The analysis of the expression the PPARg gene showed that the expression of its transcripts (NM_001001460.1) is more than four times higher in fat chickens than in lean chickens. Based on these results, we suggest that the PPARG NM_001001460.1 transcript is a potential candidate gene that highly influences the level of abdominal fat deposition in chickens.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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