High-resolution melting curve analysis of the *ADSL* and *LPL* genes and their correlation with meat quality and blood parameters in chickens

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**ABSTRACT.** Adenylosuccinate lyase (*ADSL*) and lipoprotein lipase (*LPL*) are key enzymes in the metabolism of inosine monophosphate (IMP) and fat mass, which are important factors in meat quality evaluation. In this study, we selected 50 hens from the ISA B-line layers and Guangxi Yellow chickens, slaughtered the chickens at 120 days old, and analyzed polymorphisms in the *ADSL* and *LPL* genes using the high-resolution melting curve method. Blood lipid parameters, intramuscular fat (IMF), and IMP content were higher (P < 0.05) in Guangxi Yellow chickens than in ISA B-line layers, while LPL activity was lower (P < 0.05). In exon 2 of the *ADSL* gene, a C3484T mutation was identified. In both breeds, the CC genotype showed the highest IMP, and IMP was the lowest in the TT genotype. In the 5ꞌ regulatory region of the *LPL* gene, a C293T mutation was identified. In both breeds, the CC genotype showed the lowest LPL and IMF, while IMF was the highest in the TT genotype. The percentages of individuals with the TT type in the *ADSL*
gene, which was associated with the lowest IMP, were 16.0 and 52.0% in Guangxi chickens and ISA layers, respectively. The percentages of individuals with the CC type of the \textit{LPL} gene, which was associated with the lowest LPL and IMF, were 28.0 and 44.0%, respectively. The \textit{ADSL} and \textit{LPL} gene mutations are correlated with differences in meat quality in different chicken breeds, and high-resolution melting curve is an effective prediction technology for these mutations.

\textbf{Key words:} Adenylosuccinate lyase gene; Chinese chicken breed; High-resolution melting curve technology; Lipoprotein lipase gene; Meat quality

\section*{INTRODUCTION}

The production of high-quality chicken in China is focused on meat quality. Although a standard method for evaluating meat has not been determined, differences in the meat quality of different chicken breeds are well-known. Previous studies showed that inosine monophosphate (IMP) content is an important factor in meat flavor (Manabe et al., 1991; Maga and Shahidi, 1994; Kawai et al., 2002), while intramuscular fat (IMF) content is closely related to meat tenderness, shear force, flavor, and succulence (Bejerholm and Bartongade, 1986; Eikelenboom et al., 1996; Fernandez et al., 1999). Previous studies also indicated that adenylosuccinate lyase (ADSL) catalyzes a 2-step reaction in the purine nucleotide biosynthesis pathway. ADSL has an important influence on final IMP content and plays a vital role in maintaining normal cell division and metabolism (Xu et al., 2011). Lipoprotein lipase (LPL) is a water-soluble enzyme that is synthesized and secreted by fat cells, skeletal muscle cells, and myocardial cells, but mainly by catalytic chylous particles and very low-density lipoproteins. LPL produces fatty acids and glycerol from triglycerides for cellular use and is a key enzyme in fat metabolism (Beisiegel and Heeren, 1997; Mead et al., 2002).

Single-nucleotide polymorphisms (SNPs) can be used as molecular genetic markers. Denaturing gradient gel electrophoresis, capillary electrophoresis, and TaqMan probes are currently used for SNP identification and screening. However, these methods are often subjected to low sensitivity and high false-positive rates. Direct DNA sequencing is more accurate but also more expensive. High-resolution melting curve (HRM) technology is based on efficient robust quantitative fluorescence polymerase chain reaction (PCR), without the need for a sequence-specific probe. The amplification products can be melted through precise warming, allowing for the detection of single-base differences. Thus, HRM has the advantages of being a simple, fast, and low-cost technique that provides accurate results and good repeatability (Tricarico et al., 2011). In this study, we selected 2 different chicken breeds and analyzed polymorphisms in the \textit{ADSL} and \textit{LPL} genes using HRM. Combined with the analysis of meat quality and blood lipid indicators, we discuss the molecular basis of meat quality differences.

\section*{MATERIAL AND METHODS}

\subsection*{Animals and feeding management}

Two chicken breeds, ISA B-line layers and Guangxi Yellow chickens (a domestic chick-
en breed in China), were obtained from the Guangda poultry breeding company in Zhejiang Province. ISA B-line layers (IB) had been bred for 14 generations. Guangxi Yellow chickens (GY) were collected from Rongxian County in Guangxi Province and had been bred for 10 generations. Various reproductive and body weight traits of the 2 populations are shown in Table 1.

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Body weight of cock (g)</th>
<th>Body weight of hen (g)</th>
<th>Age at first egg (day)</th>
<th>Egg production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60th day</td>
<td>Adult</td>
<td>60th day</td>
<td>Adult</td>
</tr>
<tr>
<td>IB</td>
<td>814</td>
<td>2450</td>
<td>642</td>
<td>1920</td>
</tr>
<tr>
<td>GY</td>
<td>722</td>
<td>1895</td>
<td>617</td>
<td>1495</td>
</tr>
</tbody>
</table>

All test birds were hatched on the same day and then grown indoors and grouped by breed. They were fed a maize-soybean diet that contained 11.9 megajoule/kg of metabolizable energy (ME) and 192 g/kg of Crude Protein (CP) from 1-56 days, 11.2 megajoule/kg of ME, and 158 g/kg of CP from 57-120 days.

Slaughter and sample collection

Fifty hens of each breed were randomly collected at 120 days old. From each chicken, 1.5 mL blood was collected via the wing vein and placed in a centrifuge tube containing 8 mL 70% ethanol, and then stored at -20°C for DNA analysis. Serum was collected from each chicken after coagulation of at least 1.5 mL blood at room temperature for 4 h. The sample was stored at -20°C until biochemical analysis.

The study chickens were fasted for 12 h and their live body weights were recorded. The chickens were then slaughtered and the eviscerated weight, the weights of abdominal fat, breast meat, and leg meat were measured. All weights were determined in grams. The eviscerated weight was the slaughtered body weight excluding the trachea, esophagus, crop, intestine, spleen, pancreas, gall bladder, reproductive organs, heart, liver, proventriculus, gizzard, abdominal fat, head, and feet. Breast meat weight consisted of the weights of the pectoralis major and minor muscles without the skin. Leg meat weight consisted of the weights of the thigh and drumstick without the bone and skin. Left breast muscles were stored at -20°C for IMF and IMP content determination.

Calculation of meat yields from weight data

Eviscerated yield was calculated as the ratio of eviscerated weight to live body weight after fasting. The weight percentages of the breast and leg meat were calculated as percentages of the eviscerated weight. The weight percentage of abdominal fat was calculated as a percentage of the sum of the eviscerated weight and abdominal fat weight.

Genomic DNA extraction from blood

DNA was extracted from each blood sample using a blood genomic DNA extraction kit from Axygen (Hangzhou, China).
Primer design

Primers for the ADSL gene (GenBank accession No. AY665559) and LPL gene (GenBank accession No. X60547) were as follows: ADSL-F, 5'GAAGAAGCTGCAGCAATGATGTG3'; ADSL-R, 5'CCAGCTCTGCAGCCATGATGTG3'; LPL-F, 5'GTGAAGGATTGGAGGGACAGC3'; LPL-R, 5'CCCTACAAATCAACCTGGCTCCATC3'.

PCR amplification and HRM analysis

The 15-μL PCR sample contained the following: 5.5 μL DNase-free water, 7.5 μL SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA, USA), 0.5 μL forward primer, 0.5 μL reverse primer, and 1 μL template DNA. Using the CFX 96 Quantitative PCR instrument (Bio-Rad), the amplification program was run at 98°C for 3 min, 40 cycles of denaturation at 98°C for 5 s each, and annealing/extension at 62°C for 5 s. For the product fusion program, the initial melting temperature was 75°C, followed by warming to 95°C and an increase of 0.2°C every 10 s. HRM analysis was conducted using the Precision Melt Analysis™ software (Bio-Rad).

DNA sequencing

PCR products of different genotypes were selected and the DNA was sequenced bidirectionally by the Shanghai Yingjun Biotechnology Company using an ABI 3730xl DNA Analyzer (Foster City, CA, USA).

Determination of serum biochemical index

Using an ARCHITECT C8000 automatic biochemical analyzer (Abbott Laboratories, Abbott Park, IL, USA), total cholesterol and triglyceride contents in the serum were determined. Using a DENLEY DRAGON Wellscan MK3 Elisa instrument (Thermo Scientific, Waltham, MA, USA), serum samples were analyzed for the presence of LPL enzymes.

IMF and IMP content determination

As the method introduced in NY/T 821 (MOA, 2004), fat was extracted from 10 g breast meat using a 2:3 methanol:chloroform solution (60 and 90 mL, respectively). A portion of the extract solution (50 mL) was poured into a beaker, dried at 105°C, and weighed to determine IMF content.

Two grams breast meat was added to 3.5% perchlorate solution, homogenized, and centrifuged. The supernatant was adjusted to pH 6.0 with 5 M sodium hydroxide and kept at a 100-mL constant volume. The solution was filtered through a 0.45-μm membrane. To determine IMP content, high-performance liquid chromatography was used.

Statistical analysis

SNP loci in DNA sequences were determined using the DNASTAR 7.1 software packages (DNASTAR, Inc., Madison, WI, USA). Statistical analysis was conducted using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data for traits were normally distributed. Significant
differences between traits were tested using analysis of variance and Duncan’s range tests.

RESULTS

Meat quality and blood biochemical parameters

Meat yields, quality, and blood test results are shown in Table 2. The eviscerated yield of Gangxi Yellow chickens was higher than that of ISA B-line layers (P < 0.05), while the leg meat yield was lower than that of ISA (P < 0.05). Abdominal fat yield, blood lipid parameters, and IMF content of Gangxi Yellow chickens were all much lower than those of the ISA B-line layers (P < 0.01), while LPL activity and IMP content were much higher than for ISA (P < 0.01).

<table>
<thead>
<tr>
<th>Breeds</th>
<th>EP (%)</th>
<th>AFP (%)</th>
<th>LMP (%)</th>
<th>BMP (%)</th>
<th>TC (mM)</th>
<th>TG (mM)</th>
<th>LPL (U/mL)</th>
<th>IMF (%)</th>
<th>IMP (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB</td>
<td>65.4 ± 1.8</td>
<td>1.7 ± 1.4</td>
<td>24.6 ± 1.9</td>
<td>16.2 ± 1.4</td>
<td>3.94 ± 0.70</td>
<td>1.18 ± 0.41</td>
<td>684.1 ± 237.5</td>
<td>1.39 ± 0.21</td>
<td>2.155 ± 0.303</td>
</tr>
<tr>
<td>GY</td>
<td>66.5 ± 1.7*</td>
<td>0.2 ± 0.1**</td>
<td>23.4 ± 1.9**</td>
<td>15.7 ± 1.0</td>
<td>2.87 ± 0.31**</td>
<td>0.53 ± 0.04**</td>
<td>956.7 ± 318.9**</td>
<td>1.06 ± 0.12**</td>
<td>2.597 ± 0.375**</td>
</tr>
</tbody>
</table>

EP = eviscerated yield; AFP = abdominal fat yield; LMP = leg meat yield; BMP = breast meat yield; TC = total cholesterol; TG = total triglyceride; LPL = lipoprotein lipase; IMF = intramuscular fat; IMP = inosine monophosphate. *Significant difference in means between breeds (P < 0.05); **highly significant difference in means between breeds (P < 0.01).

HRM genotype detection and DNA sequencing results

The HRM melting curve, difference curve, and classification sequence results for the ADSL gene are shown in Figure 1. In exon 2 of the ADSL gene, a C3484T mutation was identified. The HRM technique be used to can detect the CC wild-type, the CT heterologous mutation, and the TT homologous mutation.

The HRM melting curve, difference curve, and the classification sequence results for the LPL gene are shown in Figure 2. In the 5ꞌ regulatory region of the LPL gene, a C293T mutation was identified, and HRM techniques can be used to detect the CC wild-type, the CT heterologous mutation, and the TT homologous mutation.

Genotype frequency and gene frequency analysis

Gene, genotype frequency, and Homals fitness test of the 2 genes are shown in Table 3. The ADSL gene was found to be in Hardy-Weinberg equilibrium (P > 0.05), while the LPL gene deviated significantly from the Hardy-Weinberg model (P < 0.05).

Trait comparison between different genotypes

Slaughter and blood lipid parameters of the different ADSL genotypes of the 2 chicken breeds are shown in Table 4. The CC wild-type genotype had the lowest LPL activity and IMF content, while the CT type had the highest; however, the differences were insignificant (P > 0.05). IMP content between the 3 genotypes was significant. The CC type showed the highest value, while the TT type was the lowest (P < 0.05).
Figure 1. a. HRM melting curve for the *ADSL* gene. Relative fluorescence units are on the Y-axis and temperature in °C is on the X-axis. Green and red columns represent pre- and post-melting normalization regions. b. HRM difference curve for the *ADSL* gene. Difference in relative fluorescence units is on the Y-axis and temperature in °C is on the X-axis. CC genotype samples are the reference curves. c. Sequencing peak of the *ADSL* gene. The CC, CT, and TT genotypes are indicated from left to right.
HRM analysis of the *ADSL* and *LPL* genes in chickens

**Figure 2.**

(a) HRM melting curve for the *LPL* gene. Relative fluorescence units are on the Y-axis and temperature in °C is on the X-axis. Green and red columns represent pre- and post-melting normalization regions.

(b) HRM difference curve for the *LPL* gene. Difference in relative fluorescence units is on the Y-axis and temperature in °C is on the X-axis. CC genotype samples are the reference curves.

(c) Sequencing peak of the *LPL* gene. The CC, CT, and TT genotypes are indicated from left to right.
Slaughter and blood lipid parameters of the different LPL genotypes of both breeds of chickens are shown in Table 5. The CT genotype showed the lowest abdominal fat yield (P > 0.05), total cholesterol, and triglyceride content (P < 0.05), while this type had the highest LPL activity (P > 0.05). Expressions of the CC genotype were the opposite, and the CC type had the lowest IMF content and the TT type had the highest (P < 0.05).

**Table 3. Genotype and gene frequencies.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Breeds</th>
<th>Numbers</th>
<th>Genotype frequency</th>
<th>Gene frequency</th>
<th>Chi-square values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
</tr>
<tr>
<td>ADSL</td>
<td>IB</td>
<td>50</td>
<td>0.50</td>
<td>0.34</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>GY</td>
<td>50</td>
<td>0.14</td>
<td>0.34</td>
<td>0.52</td>
</tr>
<tr>
<td>LPL</td>
<td>IB</td>
<td>50</td>
<td>0.28</td>
<td>0.32</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>GY</td>
<td>50</td>
<td>0.44</td>
<td>0.26</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Significant difference between means (P < 0.05); **highly significant difference between means (P < 0.01). 
χ² 0.05 (df = 1) = 3.841; χ² 0.01 (df = 1) = 6.635.

**Table 4. Effect of ADSL genotype on traits in ISA B-line layers and Guangxi yellow chickens.**

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Genotypes</th>
<th>EP (%)</th>
<th>AFP (%)</th>
<th>LMP (%)</th>
<th>BMP (%)</th>
<th>TC (mM)</th>
<th>TG (mM)</th>
<th>LPL (U/mL)</th>
<th>IMF (%)</th>
<th>IMP (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB</td>
<td>CC</td>
<td>66.1†</td>
<td>0.15†</td>
<td>15.9†</td>
<td>22.5†</td>
<td>3.07†</td>
<td>0.60†</td>
<td>797.5†</td>
<td>1.02†</td>
<td>2.708†</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>66.5†</td>
<td>0.25†</td>
<td>15.8‡</td>
<td>23.5‡</td>
<td>2.79‡</td>
<td>0.53‡</td>
<td>1055.2‡</td>
<td>1.10‡</td>
<td>2.369‡</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>65.9†</td>
<td>0.32‡</td>
<td>16.0‡</td>
<td>24.8‡</td>
<td>4.85‡</td>
<td>1.16‡</td>
<td>641.2‡</td>
<td>1.34‡</td>
<td>2.774†</td>
</tr>
<tr>
<td>GY</td>
<td>CC</td>
<td>64.9‡</td>
<td>0.21</td>
<td>15.6‡</td>
<td>23.5‡</td>
<td>2.96‡</td>
<td>0.51‡</td>
<td>945.3‡</td>
<td>1.05‡</td>
<td>1.935*</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>65.3‡</td>
<td>0.25</td>
<td>16.0‡</td>
<td>25.1‡</td>
<td>3.80‡</td>
<td>1.01‡</td>
<td>730.0‡</td>
<td>1.34‡</td>
<td>2.648*</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>65.5‡</td>
<td>1.35</td>
<td>15.9‡</td>
<td>24.5‡</td>
<td>4.04‡</td>
<td>1.21‡</td>
<td>696.5‡</td>
<td>1.36‡</td>
<td>2.083†</td>
</tr>
</tbody>
</table>

EP = eviscerated yield; AFP = abdominal fat yield; LMP = leg meat yield; BMP = breast meat yield; TC = total cholesterol; TG = total triglyceride; LPL = lipoprotein lipase; IMF = intramuscular fat; IMP = inosine monophosphate. Small superscript letters indicate significant differences in means within the breed (P < 0.05).

**Table 5. Effect of LPL genotype on traits in ISA B-line layers and Guangxi yellow chickens.**

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Genotypes</th>
<th>EP (%)</th>
<th>AFP (%)</th>
<th>LMP (%)</th>
<th>BMP (%)</th>
<th>TC (mM)</th>
<th>TG (mM)</th>
<th>LPL (U/mL)</th>
<th>IMF (%)</th>
<th>IMP (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB</td>
<td>CC</td>
<td>66.0†</td>
<td>0.33†</td>
<td>15.6†</td>
<td>23.1†</td>
<td>2.85†</td>
<td>0.60†</td>
<td>797.5†</td>
<td>1.02†</td>
<td>2.708†</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>66.4†</td>
<td>0.21†</td>
<td>16.2†</td>
<td>23.9†</td>
<td>2.80†</td>
<td>0.49†</td>
<td>989.2†</td>
<td>1.08†</td>
<td>2.233†</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>67.1†</td>
<td>0.23†</td>
<td>15.6†</td>
<td>23.6†</td>
<td>2.90†</td>
<td>0.52‡</td>
<td>967.7†</td>
<td>1.18‡</td>
<td>2.111†</td>
</tr>
<tr>
<td>GY</td>
<td>CC</td>
<td>65.5†</td>
<td>1.35</td>
<td>16.9†</td>
<td>25.2†</td>
<td>4.16†</td>
<td>1.28‡</td>
<td>666.0‡</td>
<td>1.16‡</td>
<td>2.505*</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>65.3†</td>
<td>1.52‡</td>
<td>16.0‡</td>
<td>25.1‡</td>
<td>3.80‡</td>
<td>1.01‡</td>
<td>730.0‡</td>
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<td>2.648*</td>
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<tr>
<td></td>
<td>TT</td>
<td>65.5†</td>
<td>1.56‡</td>
<td>15.9‡</td>
<td>24.1‡</td>
<td>3.88‡</td>
<td>1.23‡</td>
<td>671.6‡</td>
<td>1.54‡</td>
<td>2.538*</td>
</tr>
</tbody>
</table>

EP = eviscerated yield; AFP = abdominal fat yield; LMP = leg meat yield; BMP = breast meat yield; TC = total cholesterol; TG = total triglyceride; LPL = lipoprotein lipase; IMF = intramuscular fat; IMP = inosine monophosphate. Small superscript letters indicate significant differences in means within breeds (P < 0.05).

**DISCUSSION**

**HRM technique and its application for detecting genotypes affecting meat quality**

HRM analysis has been used to detect genetic mutations that cause human diseases in genes such as RET, CFTR, and PEX6 (Margraf et al., 2007; Audrezet et al., 2008; Ebberink et al., 2010). Poultry infectious bronchitis and infectious bursal disease virus mutations have
been detected and reported previously (Hewson et al., 2010; Ghorashi et al., 2011). Based on our results, the HRM technique is very simple and fast, and the classification of the results is relatively clear, making HRM an ideal method for detecting SNPs in genes related to meat quality. In this study, gene amplification from only 1 mutation was detected. If more than 1 SNP is present, classification may be more complex.

Parameters associated with the ADSL and LPL genes

Shu et al. (2007) examined the ADSL gene for SNPs in exon 2 in 6 chicken breeds. Ye et al. (2010), for the Beijing You chicken, found 1 polymorphism locus (C3484T); the breast IMP content was significantly different among genotypes. Our results confirmed that C3484T mutations cause differences in IMP and that IMP content is lower in the homologous mutant TT type, which is consistent with the results of Ye.

Mu et al. (2005), using PCR-single-strand conformation polymorphism and sequence analysis on high- and low-fat of broilers for nucleotide variation in the LPL gene, found that the LPL gene mutations C235T, C278T, and C293T significantly impacted body weight and abdominal fat. In our study, only the C293T mutation was detected, which may have been related to different chicken breeds and sample numbers. The results showed that the CC type had the lowest lipoprotein lypase and intramuscular fat, while total cholesterol and triglyceride content were the highest. The LPL gene and its mutations may play an important role in regulating fat content.

Differences between chicken breeds

Meat quality and flavor are a result of interactions between components such as muscle, fat, and connective tissues. IMP content differs in different chicken breeds or lines (Huang et al., 1994; Chen et al., 2005; Tang et al., 2009); fat deposition location, content, and capacity also differ (Lv et al., 2011). Based on meat traits, GY is known for its tender and delicious meat, and IB has relatively poor meat quality as egg chicken breed. The percentages of individuals with the TT type of the ADSL gene with the lowest IMP content in GY and IB chickens were respectively 16.0 and 52.0%, while the percentages with the CC type of the ADSL gene with the highest IMP content were 50.0 and 14.0%. The percentages of individuals with the CC type of the LPL gene that produced the lowest IMF content in GY and IB chickens were respectively 28.0 and 44.0%, while the percentages with the TT type of the LPL gene that produced the highest IMF were 40.0 and 30.0%. These results relate genotype and meat quality. The detection of genes affecting meat quality may be an effective method for developing modern breeding programs.

REFERENCES


