The Identification of a Novel Transcript Variant of Chicken Lmbr1 and the Sequence Variation Analysis

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Lmbr1 has been reported to be associated with chicken polydactyly and carcass traits. Based on the hypothesis that chicken Lmbr1 should have multiple alternative splices as human and mouse Lmbr1/C7orf2, we successfully identified one novel chicken Lmbr1 transcript variant (designated as Lmbr1-2) by bioinformatics analysis and RT-PCR confirmation. Chicken Lmbr1-2 transcript was ubiquitously expressed in a range of chicken tissues. A SNP (rs14135851: G>T, also named as 288G>T reference to 603234777F1) was found to be located at the 5′ boundary (first base) of alternatively spliced exon (IN2) of chicken Lmbr1-2. Eight variants/10 haplotypes were detected from 4 breeds in a 300-bp genomic fragment surrounding 288G>T. The 288T>G and 331G>A changes were predicted resulting to the formation/loss of SRSF5 and SRSF6 motifs. The genotype/allele distribution for a G/A variant (331G>A, reference to 603234777F1) and haplotype types in polydactylous Silkie presented distinct difference with that in other four-toed breed chickens. Association analysis showed that chicken 331G>A variation had significant effect on meat quality traits including breast muscle water loss rate, leg muscle water loss rate and leg muscle fiber density, and carcass traits including evisceration weight, pancreas weight in Gushi chicken F2 resource population. These data demonstrated that chicken Lmbr1 was alternatively spliced to produce multiple splice forms as was the case in mammals, and it suggested that chicken Lmbr1-2 variation might have important effect on the carcass and meat quality traits in chicken.

Key words: chicken, Lmbr1/C7orf2, splice variant, tissue expression, variation analysis


Introduction

The nonsyndromic preaxial polydactyly (PPD) in human and mouse had been mapped to the homologous region that contains C7orf2/Lmbr1, which showed C7orf2/Lmbr1 was an important candidate gene for limb development among species (Dobbs et al., 2000; Ianakiev et al., 2001; Sato et al., 2007). Huang et al. (2006, 2007) found the significant association of Lmbr1 variation with chicken polydactylous phenotype and growth/carcass traits including shank and claw weight (SCW) and shank girth (SG) and so on. Dunn et al. (2011) mapped dominant PPD locus in Silkie (SL) chickens to a single nucleotide polymorphism (SNP) in the intron 5 of Lmbr1. A highly conserved region of intron 5 in Lmbr1/C7orf2 was identified as a cis-acting regulator of the sonic hedgehog (Shh) gene, a proposed candidate gene underlying limb abnormalities in mouse, human and chicken and was termed as “zone of polarising activity regulatory sequence” (ZRS) (Lettice et al., 2002; Goode et al., 2005; Dunn et al., 2011). Mutations in intronic ZRS region of Lmbr1 were found to be associated with ectopic Shh expression in the limb and/or polydactyly in humans, mice, cats and chickens (Gurnett et al., 2006; Masuya et al., 2007; Lettice et al., 2008; Dunn et al., 2011).

Alternative splicing is a common phenomenon of organism, which can modulate gene function, affect organismal phenotype (Cartegni et al., 2002; Lu et al., 2012). One gene often produces multiple mRNAs and protein isoforms (Wang et al., 2008). The expressions of these transcript variants may be tissue and development-stage specific (Terenzi and Ladd, 2010). Searching EST database is an effective way for finding potential splice forms (Eyras et al., 2004). The release of chicken EST database provides a great convenience for searching chicken splice variants of specific genes by informatics methods. The online chicken EST databases include NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), BBSRC ChickEST Database (http://www.chick
that mutations and chromosomal deletions/rearrangements in
et al 2011) normally spliced variant deleted 140 bp exon 4 of
codon in exon 6 (Ianakiev et al., 2001, 2006). In chicken, three
Lmbr1 transcript variants have been reported (Huang et al.,
2000, 2006). A similar abnormal splice variant deleted exon 4 of
C7orf2/Lmbr1 (and surrounding genomic region), which
introduced a reading frame shift leading to a premature stop
codon in exon 6 (Ianakiev et al., 2001). A similar ab-
normally spliced variant deleted 140 bp exon 4 of Lmbr1 has
been detected too (Huang et al., 2006). It has been inferred
that mutations and chromosomal deletions/rearrangements in
the Lmbr1 genic region may be associated with ACHP
phenotype (Lettice et al., 2003; Maas and Fallon, 2004).

Based on the hypothesis that chicken Lmbr1 should have
much more transcript variants as human and mouse, we
further identified one novel transcript isoform of Lmbr1 from
chicken EST database by EST searching and comparative
genomics analysis. Its tissue distribution patterns, the varia-
tion distributions among breeds in the surrounding retained
intron sequence, and the association between the genetic
variation and performance traits were further analyzed,
which would build a foundation to further make clear the
gene function of Lmbr1.

Materials and Methods

RNA Samples

Considering the potential spatiotemporal-specificity of
chicken Lmbr1 transcript variant, the samples from multiple
tissues/breeds/growth-stages were collected for use. The
heart tissue of one one-day-old Arbor Acres commercial
broiler and the mixed tissue samples (including heart, liver,
muscle and brain tissues) of two 7-week-old Arbor Acres
commercial broilers, two adult Silkie (SL) chickens, and two
8-week-old hybrid chickens by Gushi chicken (GS) × White
Plymouth Rock (WPR) were used for the identification of
Lmbr1 transcript variant, respectively. Fifteen tissues in-
cluding heart, liver, brain, spleen, kidney, lung, pancreas,
intestine, stomach, ovary, skin, crureus, pectoralis major,
abdominal fat and subcutaneous fat from above two 8-week-
old hybrid chickens were also used for the tissue expression
pattern analysis. The tissue samples for RNA extraction
were collected and deep-frozen in liquid nitrogen, and then
stored at −80°C. All chickens were cage-raised in Henan
Agricultural University Poultry Farms. All procedures
followed the established protocols approved by the institu-
tional animal care and use committee.

Total RNAs were extracted by Trizol reagent (Takara,
Dalian, China) according to the instructions of manufacturer
and stored at −80°C. The quantity and quality of the iso-
lated RNA was determined by GeneQuant pro ultraviolet
spectrophotometry (Amersham Pharmacia, Bucks, UK) and
agarose gel electrophoresis respectively. 1 μg total RNA
was subsequently reversely transcribed by PrimeScript® RT
reagent kit with gDNA Eraser (Takara, Dalian, China)
according to manufacturer’s instructions. The synthesized
first strand cDNA was stored at −20°C until use.

Breed Samples

In order to study the variation distribution among breeds,
blood were collected from the chicken wing vein in four
breeds including WPR (n=10), GS (n=8), SL (n=10) and
Lushi green eggshell chicken (LS, n=10), and
Lushi green eggshell chicken (LS, n=10), respectively.
Individuals were selected as unrelated as possible from each
breed. Genomic DNA was extracted from blood by phenol-
chloroform extracting method. Each breed has clearly dif-
ferent characteristics. WPR is a cosmopolitan fast-growing
breed with white plumage, which was collected from China
Agricultural University Breed Resource Conservation
Center; while SL, GS, and LS are the Chinese local breeds, which
were collected from Henan Agricultural University Poultry
Breed Resource Conservation Center. SL is an officinal
breed with black skin, feather-crest and threadlike plumage; GS is a Henan native breed, with yellow skin, yellow plum-
age and green shank. In addition, SL is a polydactylous
breed, while the others are four-toed breeds.

GS chicken F2 Resource Population

The GS chicken F2 resource population was constructed
by reciprocal crossing with GS chicken (a slow growing
Chinese native breed, 24 hens and 2 roosters ) and Anka
chicken (a fast growing broiler type, 12 hens and 4 roosters )
as Han et al. (2011, 2012) described. It includes four direct
cross-bred families (with Anka roosters mating with GS
hens) and two reciprocal families (with GS roosters mating
with Anka hens). To construct the F2 population, 9 F1
males and 1 female were selected from each of 7 families
and 1 F1 male were selected from both of 6 unrelated rooster families and 1 half sib). The F2 resource
population was established by two hatching batches at 2-
week intervals. It consists of 42 grandparents, 70 F1 parents,
and 860 F2 chickens. F2 chickens were cage raised and fed
the same corn-soybean diet with following nutritional level:
ME 11.90 MJ/kg and CP 190 g/kg from 0 to 8 weeks, then
ME 12.13 MJ/kg and CP 170 g/kg after 8 weeks. The birds
were ad libitum access to feed and water. The growth traits
were measured at different growth stages.

F2 chickens were slaughtered at the age of 84 day. Blood
were collected and carcass traits were measured. Leg muscle and breast muscle were collected for the analysis of the meat quality traits. Anticoagulated and non-anticoagulated blood samples were used for the DNA extraction and the measurement of biochemical parameters respectively. The blood genomic DNA were extracted by phenol-chloroform extracting method.

Measured growth traits include body weight (at 0, 2, 4, 6, 8, 10 and 12 week-end), shank length (SL) and shank girth (SG) at 0, 4, 8 and 12 week-end; carcass traits include body weight (BW), carcass weight (CW), semi-evisceration weight (SEW), evisceration weight (EW), abdominal fat weight (AFW), breast muscle weight (BMW), leg muscle weight (LMW), heart weight (HW), liver weight (IW), gizzard weight (GW), spleen weight (SW), pancreas weight (PW), shank length (SL) and shank girth (SW), pancreas weight (PW), shank and claw weight (SCW); meat quality traits include breast muscle water loss rate (BWLR), leg muscle water loss rate (LWLR), breast muscle fiber density (BFD), leg muscle fiber density (LFD), breast muscle fiber diameter (FBD) and leg muscle fiber diameter (FDL); serum biochemistry parameters include total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), total glycereide (TG) and serum glucose (Glu). BWLR and LWLR was calculated as follows: WLR (%)=(W1−W2)/W1×100%, where W1 and W2 are the weight of the meat sample before and after pressing. Serum TC, TG, HDL-C and LDL-C were measured by colorimetric enzymatic methods and Glu was measured by the glucose oxidase method using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Identification of Alternative Splicing Variants of Chicken Lmbr1

The chicken Lmbr1 cDNA sequence (GenBank accession no.AY251537, designated Lmbr1-N) was used to search for alternatively spliced isoforms of chicken Lmbr1 in the chicken EST database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=ncbi-blast; http://www.chick. manchester.ac.uk/; http://www.chickest.udel.edu/). The EST containing a deletion or insertion fragment (comparing with AY251537) was considered as a potential alternative splicing form of chicken Lmbr1. One EST sequence (603234777F1) that represented potential alternative splicing isoform of chicken Lmbr1 was gained from the chicken chondrocyte EST database. Based on 603234777F1 sequence, L1 primer set was designed to identify this transcript variant, whose predicted RT-PCR product length was 496 bp (Table 1, Fig. 1). To confirm the efficiency of reverse transcription, AC primer set (Table 1) was carried out to amplify a cDNA fragment of chicken β-actin (GenBank accession no. L08165). The size of PCR product for β-actin was 270 bp. PCR conditions were as follows: the initial denaturation 5 min at 94℃, then followed by 30 cycles of 30 sec at 94℃, 30 sec at 58℃, 40 sec at 72℃; and a final extension step of 7 min at 72℃. The protein structure of chicken Lmbr1 was predicted by TMPRED (Hofmann and Stoffel, 1993) and SignalP-2.0 (Nielsen and Krogh, 1998).

The Tissue Distribution of Chicken Lmbr1-2 Expression

The L1 primer set was also used to investigate the tissue distribution of chicken Lmbr1-2 isoform among 15 tissues including heart, liver, brain, spleen, kidney, lung, pancreas, intestine, stomach, ovary, skin, crureus, pectoralis major, abdominal fat and subcutaneous fat of two hybrid chickens. β-actin was used as a reference gene (with AC primer set, Table 1). A touchdown thermal cycling protocol was used for the tissue expression analysis of Lmbr1-2 and β-actin, the PCR conditions were as follows: denatured for 2 min at 94℃, followed by one cycle of 94℃ denaturation for 30 sec, 62℃ annealing for 30 sec and 72℃ extension for 40 sec; the annealing temperature was then decreased by 0.5℃ every second cycle; a further 25 cycles was carried out when the annealing temperature reached 58℃; the final extension step was 72℃ for 5 min.

Variation Detection of the DNA Fragment Surrounding rs14135851: G>T (288G>T reference to 603234777F1)

Locus in Lmbr1-2 Transcript Variant

Four breeds including WPR, GS, SL and LS were used to study the variation distribution in chicken Lmbr1 DNA fragment surrounding rs14135851: G>T variation sites with L2 primer set (reference to NW_003763661.1). L2 primer sequences were presented in Table 1 and their locations were showed in Fig. 1. The predicted PCR product length was 300 bp. PCR condition was as follows: 5 min at 94℃ for the initial denaturation, 30 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 1 min; and a final extension step of 7 min at

Table 1. The information of four primer sets

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene</th>
<th>Sequence origin</th>
<th>Forward and Reverse primer</th>
<th>Predicted size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>Lmbr1</td>
<td>603234777F1</td>
<td>F1: 5′CGGGGACTATGAAGGATGGA3′&lt;br&gt;R1: 5′TTGGTCCCTATATGGCAGT3′&lt;br&gt;</td>
<td>496</td>
</tr>
<tr>
<td>L2</td>
<td>Lmbr1</td>
<td>NW_003763661.1</td>
<td>F2: 5′CAGTTCCTGGCAGAATGAGTT3′&lt;br&gt;R2: 5′ATTGTCCAGAGCAGAAGAT3′</td>
<td>300</td>
</tr>
<tr>
<td>L3*</td>
<td>Lmbr1</td>
<td>NW_003763661.1</td>
<td>F3: 5′CAGTTCCTGGCAGAATGAGTT3′&lt;br&gt;R3: 5′ATACAGCAAAACACCAGCA3′&lt;br&gt;</td>
<td>198</td>
</tr>
<tr>
<td>AC</td>
<td>β-actin</td>
<td>L08165</td>
<td>F: 5′CTGTGTTGCCATCTCTGT3′&lt;br&gt;R: 5′CTCTGTCTCTGTTGGCT3′</td>
<td>270</td>
</tr>
</tbody>
</table>

Note: *The base presented as “C” (the 3′ end of R3 primer) is an introduced point mismatch (G→C) to create a BseNI site.
Purified PCR products were directly sequenced on ABI3730XL capillary DNA analyzer to determine the variation sites. Dnaman program was used to conduct sequence alignment. Phase program 2.1 was used to reconstruct the haplotype of each individual with the detected variation sites (Stephens and Donnelly, 2003). ESEfinder 3.0 (Cartegni et al., 2003) was used to identify the potential effect of the variants on exonic splicing enhancers (ESEs).

**Association Analysis of 331G>A Variation (reference to 603234777F1, in Fig. 1) with the Related Traits of GS Chicken F2 Resource Population**

Forced restriction fragment length polymorphism PCR (F-RFLP-PCR) method (Han et al., 2012) was used to genotype 331G>A variation in the GS chicken F2 resource population with L3 primer set, which were designed according to chicken Lmbr1 genomic sequence (NW_003763661.1). The primer sequences were presented in Table 1 and the locations were showed in Fig. 1. The PCR product length is 198 bp. In the 3’ end of the reverse primer of L3 primer set (R3), a point mismatch was introduced to create a BseNI (ACTGGN, the underlined “A” is the variation site and the underlined “G” is the mismatch base) restriction site for carriers of 331A allele, while the non-carriers of 331A allele lacked this restriction site. PCR was carried out as above described except annealing at 61°C. The 10 μL of PCR pro-
ducts were digested with 0.8 μL of BseNI (MBI Fermentas, St. Leon-Rot, Germany) overnight at 37°C, and the digested products were then separated on 3% agarose gel electrophoresis. Three genotypes were further confirmed by PCR sequencing the corresponding genotypes individuals with the forward primer of L3 (F3). For 331G>A locus, the 759 individuals of F2 chicken were genotyped by F-RFLP-PCR with BseNI enzyme.

**Statistics Model and Analysis**

Statistical program SAS 8.2 was used to analyze the relationship between the 331G>A variation and the related traits in GS chicken F2 resource population. Prior to statistical analysis, non-normally distributed traits including AFW, BW, HW and BWLR were transformed to approximate a normal distribution by logarithmical or anti-sine functions. Linear Model I with fixed effects was used to investigate the associations between genotypes and above growth traits, meat quality traits and serum biochemical parameters. Linear Model II was applied to carcass traits, with BW as a covariate to investigate its effect on carcass traits.

**Model I:**

\[ y_{ijklm} = \mu + G_i + S_j + H_k + F_l + e_{ijklm} \]

**Model II:**

\[ y_{ijklm} = \mu + G_i + S_j + H_k + F_l + b(W_{ijklm} - \bar{W})e_{ijklm} \]

Where \( y_{ijklm} \) is an observation value of the traits; \( \mu \) is the overall population mean; \( G_i \) is the fixed effect of genotype (i = 1, 3), including gene additive effect and dominant effect (additive effect with -1, 0 and 1 representing GG, AG and AA genotypes, dominant effect with -1, 0 and 1 representing GG, AG and AA genotypes); \( S_j \) is the fixed effect of sex (j = 1, 2); \( H_k \) is the fixed effect of hatch (k = 1, 2); \( F_l \) is the fixed effect of family (l = 1, 7); \( b \) is the regression coefficient for CW; \( W_{ijklm} \) is the individual CW; \( \bar{W} \) is the average CW; \( e_{ijklm} \) is the random error. All data were analyzed using a general linear model in two steps, first using a full animal model and then using a reduced animal model to exclude those non-significant effects (\( P > 0.05 \)) on the variability of traits in a preliminary analysis. The Bonferroni adjustment method was employed to compute \( p \) value for pairwise comparison among genotypes. \( P \leq 0.05 \) was considered significant. The contribution level of the 331G>A variation on phenotype variance was calculated as follows: \( \delta^2 G / \delta^2 P \), where \( \delta^2 G \) is genotypic variance, \( \delta^2 P \) is the phenotype variance.

**Results**

**Identification of Alternative Splicing Isoform Lmbr1-2**

One EST sequence (603234777F1) that represented potential alternative splicing isoform of chicken Lmbr1 was identified from the chicken chondrocyte EST database. By comparing chicken EST 603234777F1 sequence (755 bp) with chicken Lmbr1-N, it was found that chicken EST 603234777F1 sequence contained 156 bp of the 5’ UTR and 63 bp of exon 1 of chicken Lmbr1-N. The remaining 536 bp sequence at the 3’-end was aligned with IN-1 (68 bp) and IN-2 (468 bp) fragments of chicken Lmbr1-N intron 1 respectively, about 1600 bp apart. A pair of specific primers (L1 primer set) whose forward primer is positioned at 5’ UTR and the reverse primer is positioned at IN-2 sequence (Fig. 1), was designed to further verify this transcript. One 428 bp specific PCR product (confirmed by agarose gel electrophoresis and PCR product sequencing) was generated from the heart cDNA of one-day-old broiler. A comparison indicated that this transcript was another novel splice variant of chicken Lmbr1 (designated Lmbr1-2) deleted 68 bp IN-1 fragment of EST 603234777F1, which was predicted to encode a 30 amino acid (AA) protein (Fig. 1). The Lmbr1-2 nucleotide and predicted protein sequence have been submitted to GenBank (GenBank accession no. EF682501 and ABU82890). A rs14135851: G>T variant (288G>T in Fig. 1, reference to 603234777F1) was found to be positioned at the 5’ boundary of IN-2 (first base) and predicted to cause a Gly/Cys amino acid change in transcript variant Lmbr1-2 (Fig. 1). The allele of this site was G in our RT-PCR sequencing result and EST 603234777F1 (Fig. 1). Chicken Lmbr1-N (AAP04355.2) was predicted containing nine transmembrane domains and no signal sequence with N-terminus of the protein in the cytoplasm and the C-terminus facing the lumen (Clark et al., 2000); While Lmbr1-2 protein was predicted not containing transmembrane domains (http://www.ch.embnet.org/software/TMPRED_form.html; http://www.cbs.dtu.dk/services/SignalP-2.0/). In the mouse, there was a similar short transcript (AF190666, BB614787) as chicken Lmbr1-2 transcript variant, which included a 286-bp intron 1 fragment of the mouse Lmbr1 long transcript (AF190665) and was predicted to encode a 32 AA protein (Clark et al., 2000). The similarity of Lmbr1-2 alternative splice between species shows the potential importance of this transcript.

By detecting the RT-PCR products of L1 primer set with agarose gel electrophoresis and PCR sequencing respectively, it was found that transcript variant Lmbr1-2 (with G allele at 288G>T locus) was detected in all mixed tissue samples from two 7-week-old Arbor Acres commercial broilers, two adult SL chickens and two 8-week-old hybrid chickens, while transcript variant EST 603234777F1 (retained IN-land IN-2) was not detected in any samples.

**Variation of the DNA Fragment Surrounding rs14135851: G>T (288G>T 603234777F1) Locus in Lmbr1-2 Transcript Variant**

Further analyzing the variation distribution of the DNA fragment surrounding rs14135851: G>T (288G>T in Fig. 1) locus with L2 primers, eight variants were detected from four breeds and submitted to dbSNP database (Table 2), their corresponding NCBI ss# number were presented in Table 2. The frequency of 288T allele was low in whole population (0.13). LS, WPR and GS contain 7, 6 and 4 variants respectively, while SL only contains two variants (Table 2). Ten haplotypes were reconstructed by Phase program with eight variation sites. LS, WPR and GS contain 7, 6 and 5 haplotypes respectively, while SL only contains two haplotypes (Table 2). ESEfinder 3.0 predicted that 288T and 331G allele contain a SRSF6 motif (”TGTAGA” for 288T and “TGTGGC” for 331G) respectively, while 288T→G and 331G→A change could result to the loss of above SRSF6
motifs and the formation of SRSF5 motifs ("TTTCAGG" for 288G and "TGACTGC" for 331A) respectively (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi).

The Tissue Expression of Chicken Lmbr1-2 Transcript Variant

By RT-PCR method, we analyzed the expression profiling of chicken Lmbr1-2 transcript variant in 15 tissues of two hybrid chickens. It showed that chicken Lmbr1-2 was expressed in 15 analyzed tissues including heart, liver, brain, spleen, kidney, lung, pancreas, intestine, stomach, ovary, skin, crureus, pectoralis major, abdominal fat and subcutaneous fat, and the expression of chicken Lmbr1-2 had not showed clear tissue difference among 15 analyzed tissues (Fig. 2).

The Association Analysis of 331G > A Variation (reference to EST 603234777F1) with the Related Traits of GS Chicken F2 Resource Population

Three genotypes were detected from GS chicken F2 resource population by F-RFLP-PCR method with BseNI enzyme for 331G > A variation, and denoted as GG genotype (198 bp), AA genotype (177 bp and 21 bp) and AG genotype (198 bp, 177 bp and 21 bp) respectively. The 21 bp fragment was too short to be visible by 3% agarose gel electrophoresis. Allele 331A is the minor allele in F2 population, whose frequency was 15.54%.

We conducted the association analysis of 331G > A variation with above growth traits, carcass traits, meat quality traits and serum biochemical parameters in GS chicken F2.
resource population. With three genotypes (AA, AG and GG), it was found that 331G>A variation were significantly associated with LWLR, LFD, SEW, PW and LDL-C ($p < 0.05$). After Bonferroni correction, the AG genotype value for BWLR ($p < 0.05$) and LWLR ($p < 0.05$) was significantly lower than that GG genotype value (Table 3). The contribution levels of three genotypes for traits were presented in Table 3. The analysis to the genetic effect on the above traits shows that the additive effect was significant for SEW and LDL-C traits ($p < 0.05$), dominant effect was significant for PW, LFD and LDL-C ($p < 0.05$). In addition, 331G>A variation had an overdominant effect on both LWLR and LFD (Table 4).

Considering the small sample number of AA genotype ($n = 16$) in F2 population, we further excluded AA genotype individuals and analyzed the relationship between 331G>A variation and the related traits, it was found 331G>A variation had extremely significant effect on BWLR and LWLR ($p < 0.01$), and significant effect on SEW, PW and LFD ($p < 0.05$) respectively (Table 3).

**Discussion**

Based upon the observed levels of conservation, Thanaraj *et al.* (2003) used a transcript coverage model to extrapolate that 74% of constitutive human splice junctions and 61% of alternative human splice junctions are conserved in the mouse genome. With the increase of EST database, bioinformatics was an effective ways for identification of alternative splice forms (Thanaraj *et al.*, 2003; Kan *et al.*, 2004). Multiple Lmbr1 transcript isoforms have been identified from human, mouse and chicken, and several of the alternative splicing forms were found to be conservative among species (Clark *et al.*, 2000; Ianakiev *et al.*, 2001; Huang *et al.*, 2006, 2011). This indicated that genomic comparison should be another effective way of identifying alternative isoforms (Thanaraj *et al.*, 2003; Kan *et al.*, 2004). Until now, four splice isoforms of chicken Lmbr1 were identified, which showed that chicken Lmbr1 also had multiple alternative splicing forms, as was the case in mammals. Comparison of the four identified transcripts of chicken Lmbr1 (reference to chicken Lmbr1-N) shows that the alternative splicing forms of chicken Lmbr1 include intron retention (Lmbr1-2), initiation site change by alternative 5' splice site (Lmbr1-1) (Huang *et al.*, 2011), and exon deletion (Huang *et al.*, 2006). Lmbr1-2 and EST 603234777F1 may represent two different alternative splice variants of chicken Lmbr1, but here we failed to find the transcript variant as EST 603234777F1 from multiple breeds/tissues/stages. This

<table>
<thead>
<tr>
<th>Traits</th>
<th>AA ($n=16$)</th>
<th>AG ($n=204$)</th>
<th>GG ($n=539$)</th>
<th>$^{1}$p value</th>
<th>$^{2}$p value</th>
<th>Contribution level for traits (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEW (g)</td>
<td>1160.41±60.73</td>
<td>1098.77±13.64</td>
<td>1093.77±8.07</td>
<td>0.0429</td>
<td>0.0456</td>
<td>1.63%</td>
</tr>
<tr>
<td>PW (g)</td>
<td>3.53±0.17</td>
<td>3.23±0.05</td>
<td>3.35±0.30</td>
<td>0.0303</td>
<td>0.0424</td>
<td>2.14%</td>
</tr>
<tr>
<td>BWLR (%)</td>
<td>23.83±1.25</td>
<td>23.36±0.37</td>
<td>24.44±0.23</td>
<td>0.0466</td>
<td>0.009</td>
<td>1.41%</td>
</tr>
<tr>
<td>LWLR (%)</td>
<td>17.14±1.07</td>
<td>15.82±0.31</td>
<td>16.51±0.20</td>
<td>0.027</td>
<td>0.009</td>
<td>1.01%</td>
</tr>
<tr>
<td>LFD (fibres/mm$^2$)</td>
<td>1067.82±80.27</td>
<td>902.83±23.62</td>
<td>965.92±14.63</td>
<td>0.0395</td>
<td>0.0311</td>
<td>2.29%</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>1.26±0.11</td>
<td>1.00±0.03</td>
<td>1.06±0.02</td>
<td>0.0394</td>
<td>0.162</td>
<td>2.10%</td>
</tr>
</tbody>
</table>

Values represent LSM±SE
Abbreviations: SEW, semi-evisceration weight; PW, pancreas weight; BWLR, breast muscle water loss rate; LWLR, leg muscle water loss rate; LFD, leg muscle fiber density; LDL-C, lipoprotein cholesterol.

$^{1}$p value, the p value of genotype effect on traits analyzed with three genotypes; $^{2}$p value, the p value of genotype effect on traits analyzed with two genotypes (AG and GG).

$^{a,b}$ Means with no same superscript differ significantly among three genotypes after Bonferroni adjustment ($p<0.05$).
may be related with its high spatio-temporal expression specificity or its expression is too weak to be detected by this method.

*C7orf2/Lmbr1* was an important gene for limb development among species (Ianakiev et al., 2001; Huang et al., 2006; Sato et al., 2007). The dominant PPD locus was mapped to the ZRS region (Intron 5 of *Lmbr1*), and the ZRS SNP is associated with allelic imbalance of Shh expression in the limbs, with Shh preferentially expressed from the SL chicken Shh allele in ZRS SNP heterozygotes (Dunn et al., 2008). The 288T variant was positioned at the 5′ boundary (first base) of alternative spliced exon IN-2. Whether 288T>G and 331G>A variation contribute to the *Lmbr1* transcript isoforms with the expression difference among breeds/individuals need to be further identified.

It seems that chicken *Lmbr1* have pleiotropic effects. It has also been reported that chicken variation in exon 16 (c.1254T>C) was significantly associated with chicken carcass traits including EW (*P*<0.01), GW (*P*<0.01), SCW (*P*<0.01) and SG (*P*<0.01) traits in CAU F2 resource population established by F2 crossing of SL chicken and WPR broilers (Huang et al., 2007). Here, it revealed that chicken 331G>A variation (reference to 603234777F1) had significant effect on SEW (*P*<0.05) and PW (*P*<0.05) in GS F2 resource population. The SEW is carcass weight removing chicken heart, liver, head and neck, wing, shank and claw, gizzard, glANDar stomach and abdominal fat. The significant association of two *Lmbr1* variants (331G>A reference to 603234777F1 and c.1254T>C) with SEW and EW in different F2 resource population shows the potential effect on carcass traits.

In all F2 individuals of GS F2 resource population, some important meat quality traits were measured, which provide a great convenience for to further learn about the effect of genetic variation on these traits. BWLR and LWLR are the traits reflecting muscle water holding capacity, which are the important property of fresh meat as they affect both the yield and the quality of the end product (Rao et al., 1989). The genetic variation (such as halothane gene in pig) may had important effect on muscle water holding capacity (Huff-Lonergan and Lonergan, 2005; Wariththiam et al., 2010),

### Table 4. Genetic effect of 331G>A variant (reference to 603234777F1, see Fig. 1) of chicken Lmbr1

<table>
<thead>
<tr>
<th>Traits</th>
<th>BA A</th>
<th>p value</th>
<th>BD D</th>
<th>p value</th>
<th>Root MSE</th>
<th>$\sigma^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEW(g)</td>
<td>38.64</td>
<td>0.05</td>
<td>13.50</td>
<td>0.24</td>
<td>155.96</td>
<td>52.84</td>
</tr>
<tr>
<td>PW(g)</td>
<td>0.13</td>
<td>0.12</td>
<td>0.13</td>
<td>0.01</td>
<td>0.67</td>
<td>0.09</td>
</tr>
<tr>
<td>BWLR (%)</td>
<td>−0.49</td>
<td>0.44</td>
<td>0.39</td>
<td>0.29</td>
<td>4.95</td>
<td>−0.31</td>
</tr>
<tr>
<td>LWLR (%)</td>
<td>0.29</td>
<td>0.59</td>
<td>0.53</td>
<td>0.08</td>
<td>4.20</td>
<td>0.32</td>
</tr>
<tr>
<td>LFD (fibers/mm²)</td>
<td>45.68</td>
<td>0.26</td>
<td>57.33</td>
<td>0.01</td>
<td>294.73</td>
<td>50.95</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>0.11</td>
<td>0.05</td>
<td>0.08</td>
<td>0.01</td>
<td>0.38</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Abbreviations: SEW, semi-evisceration weight; PW, pancreas weight; BWLR, breast muscle water loss rate; LWLR, leg muscle water loss rate; LFD, leg muscle fiber density; LDL-C, lipoprotein cholesterol; BA, regression coefficients of additive effect (A); BD regression coefficients of dominant effect (D).

$\sigma^2$ = gene substitute effect = a + d(q−p), a = (AA−GG)/2, d = (GG+AA)/2, p and q is allelic frequencies of allele G (84.46%) and allele A (15.54%) respectively.
and it was reported that beef muscle water holding capacity was strongly positive correlated with muscle fiber diameter (Rao et al., 1989). Here 331G>A variant shows significant association with BWLR, LWLR and LFD. The heterozygous genotype (AG) was the advantage genotype; its value was significantly lower than that GG genotype for BWLR, LWLR and LFD traits respectively. It shows the potential effects of Lmbr1 variation on these traits, and the correlation among them.

It has been reported in human that 52% to 80% alternative splicing events are regulated between tissues, which provide an important element of support for the hypothesis that alternative splicing is a principal contributor to the evolution of phenotypic complexity (Pan et al., 2008; Wang et al., 2008). As a limb development related gene, Lmbr1-2 is ubiquitously expressed in a range of chicken tissues, which shows that it probably plays an important role during growth and development of multiple organs (Trakooljul et al., 2010). Future work will be to further unravel the alternative splicing mechanism behind these, and to understand the functions of Lmbr1.

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