

## Research Article

# Developmental Stage-Specific Imprinting of *IPL* in Domestic Pigs (*Sus scrofa*)

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Imprinted in placenta and liver (*IPL*) gene has been identified as an imprinted gene in the mouse and human. Its sequence and imprinting status, however, have not been determined in the domestic pigs. In the present study, a 259 base pair-specific sequence for *IPL* gene of the domestic pig was obtained and a novel SNP, a T/C transition, was identified in *IPL* exon 1. The C allele of this polymorphism was found to be the predominant allele in Landrace, Yorkshire, and Duroc. The frequency of CC genotype and C allele are different in Duroc as compared with Yorkshire ( $P = .038$  and  $P = .005$ , resp.). Variable imprinting status of this gene was observed in different developmental stages. For example, it is imprinted in 1-day-old newborns (expressed from the maternal allele), but imprinting was lost in 180-day-old adult (expressed from both parental alleles). Real-time PCR analysis showed the porcine *IPL* gene is expressed in all tested eight organ/tissues. The expression level was significantly higher in spleen, duodenum, lung, and bladder of 180-day-old Lantang adult compared to that in 1-day-old newborns Lantang pigs ( $P < .05$ ). In conclusion, the imprinting of the porcine *IPL* gene is developmental stage and tissue specific.

## 1. Introduction

Genomic imprinting is an unusual epigenetic phenomenon in which the allele inherited from one parent is epigenetically silenced in the offspring and escapes the Mendel's laws of heredity [1, 2]. Previous studies have demonstrated that genomic imprinting was implicated in the regulation of the placental and fetal weight in mice [3]. It has also been shown to contribute to the body composition of the pig [4]. These observations indicated the apparent importance of genomic imprinting in the process of growth and development.

*IPL* (imprinted in placenta and liver) gene is also known as *PHLDA2* and *TSSC3* [5] and may play an important role in the regulation of fetal overgrowth and growth retardation [3, 6]. As the name implies, *IPL* is imprinted in yolk sac, placenta, and fetal liver, and in adult spleen and rib. Biallelic expression of *IPL* has been found in the adult heart, lung, liver, kidney, brain, and intestine of the mouse [7],

suggesting that the imprinting status of *IPL* is organ/tissue and developmental stage specific.

The imprinting status of genes can be determined by tracing the expression pattern of genetic markers in the offspring. One of the most frequently used genetic markers is single nucleotide polymorphism (SNPs). The present study was designed to examine the imprinting status of porcine *IPL* in different organ/tissues and various developmental stages. To this end, we subcloned the partial sequence of domestic pigs *IPL* gene, identified an SNP in *IPL* exon1, investigated the allelic and genotypic frequencies in different domestic pigs breeds, analyzed the imprinting status in twelve organ/tissues of newborn and adult pigs, and examined relative expression levels of *IPL* gene in eight organ/tissues of newborns and adult Lantang and Landrace pigs. These results fill in the knowledge gap of genomic imprinting in species other than the human and mouse and are important references for studies of the abnormalities

of animals produced from assisted reproduction. A greater understanding of gene imprinting in development is essential for investigations into disruptions of these imprinting patterns under circumstances of assisted reproduction.

## 2. Materials and Methods

**2.1. Organ/Tissues Samples and Isolation of Genomic DNA and Total RNA.** Ear or tail samples were obtained from 230 pigs from the Guangdong BaiLing Pig Breeding Farm. DNA was extracted from these homogenized samples by proteinase K (0.2 mg/mL) digestion, followed by conventional phenol-chloroform extraction and sodium acetate/ethanol precipitation. The DNA samples were used to identify SNPs, heterozygosity screening, and allele frequency determination. For allelic determination of *IPL* expression, organ/tissue samples, including the heart, liver, brain, spleen, kidney, lung, stomach, pancreas, thymus, bladder, muscle, placenta, and tongue, were obtained from 6 heterozygous pigs (3 1-day-old newborns and 3 180-day-old adults) whose mothers are homozygous for the SNP. To assess the expression level of this gene, eight organ/tissue samples, including the muscle, liver, stomach, hypothalamus, spleen, duodenum, lung, and bladder, were obtained from twelve Landrace pigs (6 1-day-old and 6 180-day-old) and twelve Lantang (6 1-day-old and 6 180-day-old). All organ/tissue samples for extracting total RNA were frozen immediately after collection and stored at  $-80^{\circ}\text{C}$  until use. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (TaKaRa Bio Inc.) to remove any contaminating genomic DNA from these organ/tissue samples.

**2.2. Molecular Cloning of the *IPL* Gene in Domestic Pigs.** Highly conserved ESTs were obtained through standard BLAST analysis according to partial sequences of the human (AF001294) and mouse (AF002708) *IPL* genes. Gene-specific primers for *IPL* gene were designed using the highly conserved ESTs. The primers were as follows: S071, 5'-GCG AGG GCG AGT TGG AGA AGC G-3' and S072, 5'-GAT GGC CGC GCG TTC CAG CAG CT-3'. The PCR products from amplifying genomic DNA were then subcloned in PMD<sub>18</sub> T vectors (TaKaRa Bio Inc.) and directly sequenced on ABI PRISM model 3700 (Applied Biosystems, Foster City, CA). A 259 bp fragment was obtained by sequencing and has been found to locate in exon 1 of the porcine *IPL* gene.

**2.3. Identification of SNP of *IPL* Gene and Determination of Allele and Genotype Frequencies of this SNP.** Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and PCR fragment length polymorphism (PCR-RFLP) were used to identify SNP in the pig *IPL* gene and genotyping of this SNP [8–10]. The primers were as follows: S075, 5'-CCC CGA CCG CCT GAG CCT T-3' and S076, 5'-GCG CAG CGG AAG TCG ATC TC-3'. Each PCR reaction was performed in 25  $\mu\text{L}$  containing 50 ng genomic DNA, 0.6 pmol/ $\mu\text{L}$  primer S075, 0.6 pmol/ $\mu\text{L}$  primer S076, and 0.2  $\mu\text{L}$  Taq DNA polymerase (TaKaRa Bio Inc.). The following amplification conditions were used: an initial

denaturation step of  $94^{\circ}\text{C}$  for 2 minutes was followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 seconds, annealing at  $61^{\circ}\text{C}$  for 30 seconds, and extension at  $72^{\circ}\text{C}$  for 10 seconds, finishing hold at  $72^{\circ}\text{C}$  for 5 minutes. The PCR products were denatured at  $99^{\circ}\text{C}$  for 10 minutes, chilled on ice immediately, and conducted on 12% polyacrylamide gels, run in a cold room ( $4^{\circ}\text{C}$ ) at 200 V for approximately 12 hours. The present of different banding patterns on SSCP for exon1 indicated the existence of one SNP. The PCR products of three animals with different banding patterns were directly sequenced to identify the nucleotide change at the SNP locus. The SNP locus included a site of restriction Enzyme TSPR I (New England Biolabs, inc.) after the analysis of primer 5.0 software. For RFLP, 8  $\mu\text{L}$  PCR products were digested by restriction enzyme TSPR I (New England Biolabs, inc.) at  $65^{\circ}\text{C}$  overnight, then separated by 3% agarose gel electrophoresis. Allele and genotype frequencies were estimated by direct counting.

**2.4. Allele-Specific Expression of *IPL* in Domestic Pigs.** The cDNAs were made from total RNA from organ/organ/tissue samples of three heterozygous piglets and adult pigs. PCR products from S075 and S076 were subjected to PCR-RFLP to analyze allele-specific expression of *IPL*.

**2.5. Organ/Tissue-Specific Expression Levels of *IPL* Gene in Domestic Pigs.** A SYBR green (TaKaRa Biotech, Dalian, China)-based real-time RT-PCR for the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Inc.) was used for analyzing the organ/tissue-specific expression levels of *IPL*. Because of the excellently stable expression of  $\beta$ -actin across different pig tissues [11], this gene was chosen as the internal reference gene in this study. The primer sequences for  $\beta$ -actin were as follows: S077, 5'-TGC GGG ACA TCA AGG AGA A-3' and S078, 5'-TCG TTG CCG ATG GTG ATG-3'. Primers S075 and S076 were used for analyzing the expression of *IPL*. The cDNA was made from total RNA from each organ/tissue sample. The following amplification conditions for  $\beta$ -actin and *IPL* were used: denaturation step at  $95^{\circ}\text{C}$  for 1 minute, 40 cycles were chosen at  $95^{\circ}\text{C}$  for 15 seconds,  $58^{\circ}\text{C}$  for 15 seconds, and  $72^{\circ}\text{C}$  for 45 seconds. The relative amount of *IPL* gene =  $2^{-\Delta\Delta\text{Ct}}$ , where  $\Delta\text{Ct} = \text{Ct}_{IPL} - \text{Ct}_{\beta\text{-actin}}$  and  $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{calibrator}}$ . The calibrator chosen for this study was a pooled sample of RNA from all eight organ/tissues of pigs [12]. Validation of the amplification efficiency of *IPL* and  $\beta$ -actin was completed for each gene before using the  $2^{-\Delta\Delta\text{Ct}}$  method for quantification. Briefly, Serial dilutions of cDNA were amplified by real-time PCR using gene-specific primers. The specific amplification of *IPL* gene was detected by dissociation curve (Figure 1(a)). The amplification efficiencies of *IPL* and  $\beta$ -actin were close to 100% (0.995 for  $\beta$ -actin and 0.95 for *IPL*) and were plotted using least squares linear regression analysis (Figure 1(b)). Each sample analysis was conducted in triplicate.

**2.6. Statistical Analysis.** The levels of *IPL* expression were compared using Student's *t*-test using SPSS version 12.0 for Windows (SPSS Inc, Chicago, IL, USA).  $P < .05$  was considered statistically significant.

TABLE 1: Allelic and genotypic frequencies of the domestic pigs *IPL* gene.

Genotype Allele	Landrace ( <i>N</i> = 90)	Yorkshire ( <i>N</i> = 50)	Duroc ( <i>N</i> = 90)	<i>P</i> <sup>a</sup> value	<i>P</i> <sup>b</sup> value	<i>P</i> <sup>c</sup> value
CC (%)	49 (54.4)	33 (66.0)	43 (47.8)	.184	.371	.038
TT (%)	35 (38.9)	14 (28.0)	40 (44.4)	.196	.450	.055
CT (%)	6 (6.7)	3 (6.0)	7 (7.8)	.878	.773	.696
C (%)	104 (57.8)	69 (69.0)	93 (51.7)	.064	.244	.005
T (%)	76 (42.2)	31 (31.0)	87 (48.3)	.064	.244	.005

*P*<sup>a</sup> value: Landrace versus Yorkshire; *P*<sup>b</sup> value: Landrace versus Duroc; *P*<sup>c</sup> value: Yorkshire versus Duroc.

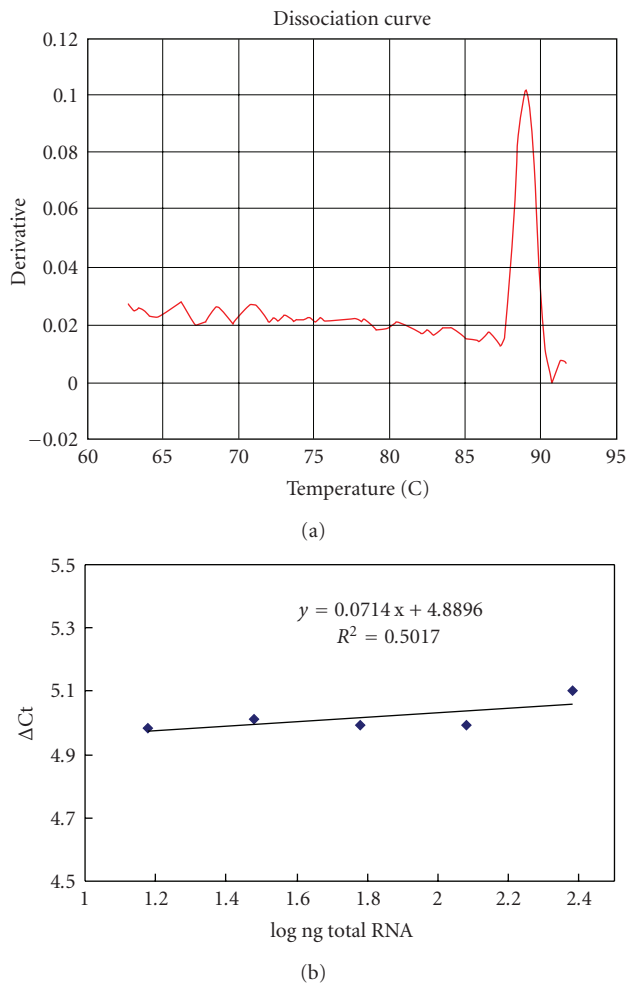


FIGURE 1: A representative validated curve of real-time PCR of *IPL* gene. (a) The dissociation curve of PCR products of *IPL* gene. Only one dissociation peak was observed, demonstrating that the amplification is specific. (b) The validation curve for multiple dilutions of RNA. The slope rate of the curve was  $<0.1$ , demonstrating that the same amplified efficiency has been achieved from different concentration of *IPL* mRNA.

### 3. Results

**3.1. Cloning of Domestic Pigs *IPL* Gene.** In the present study, we obtained a 259 bp partial sequence of the domestic pig *IPL* gene. This sequence is located in exon 1 of *IPL*. Our result showed that the domestic pigs *IPL* gene exon1 is high in GC

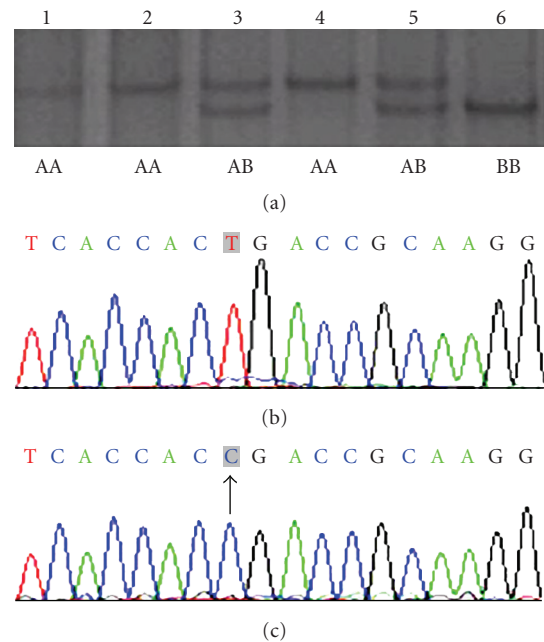


FIGURE 2: Identification of an SNP in the domestic pigs *IPL* gene by PCR-SSCP and sequencing. (a) SSCP image of three different banding patterns of PCR products, demonstrating that an SNP was observed in exon1 of *IPL* gene. (b) and (c) Chromatographs of the domestic pigs *IPL* gene near the SNP in exon1 of *IPL* gene showing TT and CC genotype homozygote.

content (66.8%). The homology of the porcine *IPL* sequence is 95% and 84% to that of humans and the mouse.

**3.2. Identification of SNP in Domestic Pigs *IPL* Exon1 and Allelic and Genotypic Frequency of the SNP.** To identify a transcribed polymorphism in *IPL* gene, primer S075 and primer S076 were used to amplify exon 1 from genomic DNA. An SNP was identified by SSCP analysis (Figure 2(a)). The polymorphism is a T/C transition at the 200th nucleotide (from 5' to 3') of cDNA sequence (Figure 2(b)). To determine the frequency of each allele (T or C) of the SNP, we analyzed 230 DNA samples from Landrace, Duroc and Yorkshire pigs. The result was shown in Table 1. The C allele was the predominant allele in all breeds tested. The distribution of the genotype frequencies was the highest for CC, medium for TT, and the lowest for CT. The frequencies of the CC genotype and the C allele are different in Duroc as compared with Yorkshire ( $P = .038$  and  $P = .005$ , resp.).

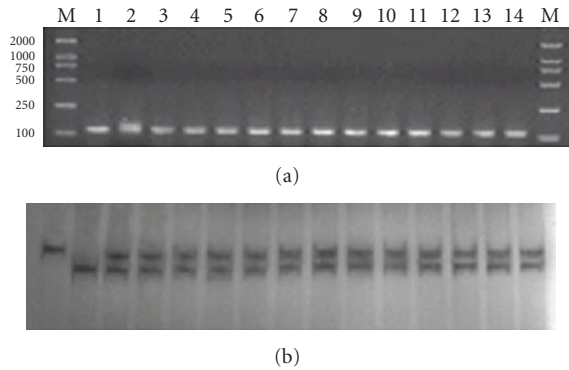


FIGURE 3: PCR-RFLP or SSCP image of the allelic expression of domestic pigs *IPL* gene. (a) Allelic expression of 1-day-old domestic pig using PCR-RFLP method: Lanes M, molecule marker; Lanes 1 and 2, genotypes of the female parent and the newborn; lanes 3–14, expression patterns of heart, liver, brain, spleen, kidney, lung, stomach, pancreas, thymus, bladder, muscle and placenta of piglet. (b) Allelic expression of 180-day-old domestic pig using PCR-SSCP method: Lanes M, molecule marker; Lanes 1 and 2, genotypes of the female parent and the adult domestic pig, lanes 3–15, expression patterns of heart, liver, brain, spleen, kidney, lung, stomach, pancreas, thymus, bladder, muscle, placenta and tongue of piglet.

**3.3. Allelic Expression of the Porcine *IPL* Gene.** Six heterozygous pigs, 3 at one day of age and 3 at 180 days of age, were used to determine whether *IPL* gene is imprinted in domestic pigs. All twelve organ/tissue samples including the heart, liver, brain, spleen, kidney, lung, stomach, pancreas, thymus, bladder, muscle, and placenta from the 1-day-old newborn pigs showed monoallelic expression of the maternal allele (Figure 3). However, biallelic expression of *IPL* was found in 180-day-old domestic pigs, suggesting a loss of imprinting in adult domestic pigs.

**3.4. Expression Levels of the Porcine *IPL* Gene.** To assess the levels of *IPL* expression in a tissue-and stage-specific patterns, real-time PCR was performed in eight organ/tissues including muscle, liver, stomach, hypothalamus, spleen, duodenum, lung, and bladder of 1-day and 180-day-old pigs (Figures 4(a) and 4(b)). Higher levels of *IPL* expression were observed in 180-day-old than in 1-day-old Landrace and Lantang pigs in spleen, duodenum, lung, and bladder (Figures 4(a) and 4(b)).

## 4. Discussion

In the present study, our results showed that *IPL* exhibits monoallelic expression of the maternal allele in newborn and biallelic expression in adult domestic pigs. Meanwhile, *IPL* expression level is higher in adults than in newborns.

By first obtaining the partial exon 1 sequence, we found that, typical to imprinted genes [13–15], the porcine *IPL* gene was highly GC rich in the exon1 region. Using the expressed SNP (C/T transition) in exon 1 identified in this study, we discovered different allelic frequency of this SNP in

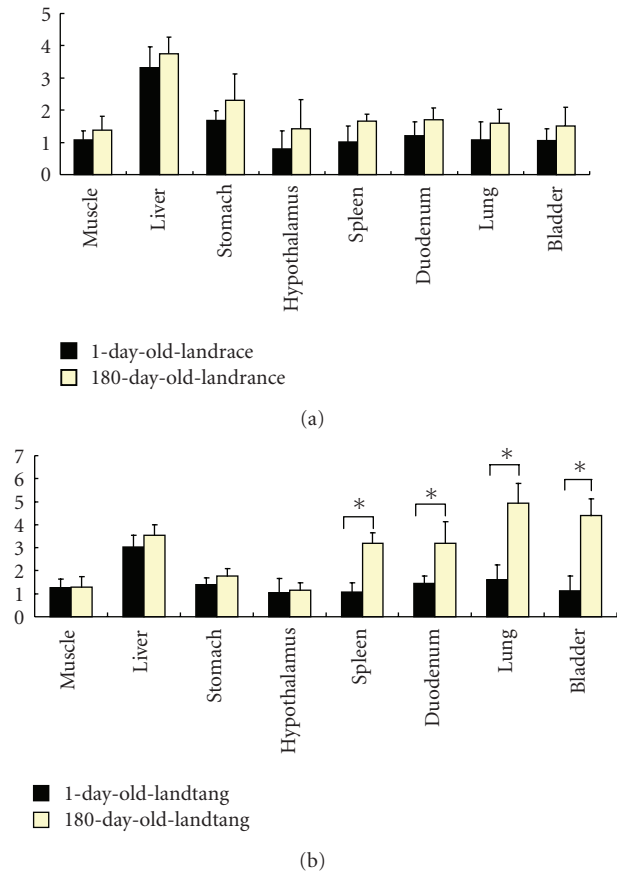


FIGURE 4: Relative expression levels of *IPL* gene in muscle, liver, stomach, hypothalamus, spleen, duodenum, lung, and bladder of Landrace and Lantang pigs. (a) the expression level of *IPL* in eight organ/tissues of Landrace pigs. (b) the expression level of *IPL* in eight organ/tissues of Lantang pigs. \*significant difference ( $P < .05$ ).

different pig breeds (Yorkshire versus Duroc), which could be a result of selective breeding in modern agriculture. It is also worthwhile to point out that heterozygosity was low in our tested population and the genotype frequency of these three breeds represented a large deviation from Hardy-Weinberg Equilibrium ( $P < .000001$ ) and it can be explained by gene drift due to selective breeding in small populations. The identification of an expressed SNP in *IPL* gene also allowed us to study its imprinting status by following the expression of the parental alleles in heterozygous pigs. We found that *IPL* is imprinted in all organ/tissues studied in 1-day-old pigs. These observations are consistent with those reported by Qian et al. [7] who documented that the human *IPL* gene was imprinted in the placenta and liver, and in all fetal organ/tissues in mice. Furthermore, *IPL* was also reported to be imprinted in fetal kidney and intestine in humans [16]. Interestingly, our result showed that imprinting of *IPL* was lost in all organ/tissues tested in 180-day-old adult domestic pigs. This result is also consistent with the observations reported by Muller and coworkers who found loss of imprinting of *IPL* in human normal, adult brain and blood [17].

Previously, Qian et al. [7] found that the expression of *IPL* is developmental stage specific in the mouse. Similarly, our results also showed that the higher expression of *IPL* was found in adult domestic pigs. The fact that we also observed loss of *IPL* imprinting in adults could explain the increased levels of gene expression because transcription occurs from both the maternal and paternal alleles instead of only from the maternal alleles in the newborns.

Interestingly, we also observed that the expression levels of *IPL* were different between Landrace and Lantang pigs. One possible explanation is that traits such as production and growing performance are differentially selected in different pig breeds. Because *IPL* is a growth-regulating gene, the difference in *IPL* expression levels in different breeds is one of the underlying mechanisms of such growth properties. Similar observations such as breed-typical gene expression profiles in the liver have been reported previously [18].

Although the imprinting status of *IPL* has been well documented in the mouse and human and *IPL*'s function in the control of placental growth has been demonstrated in mouse knockout models [3, 6], the detailed mechanisms of its epigenetic regulation have not been fully understood. Conflicting reports on the role of DNA methylation on *IPL* imprinting are found in the literature. McMinn et al. [19] reported that altered expression of *PHLDA2* was not accompanied by changes in DNA methylation within its imprinting center, while Li et al. [20] observed a correlation between upregulated expression of *PHLDA2* gene and hypomethylation of its promoter region. Additionally, Diplas et al. [21] reported that mechanisms other than loss of imprinting may contribute to dysregulation of the *PHLDA2* gene. Further studies are needed to elucidate whether changes in DNA methylation are associated with loss of imprinting of *IPL* in the pigs, such studies in a new species are necessary to clarify the conflicting observations in mice and humans.

In conclusion, this is the first study on the imprinting status of *IPL* in species other than the mouse or human. Our results showed that there is a general conservation of imprinting and loss of imprinting for *IPL/PHLDA2* in all three species that have been studied so far, suggesting that the imprinting of this gene is likely established before the divergence of these species more than 90 million years ago [22].

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