Short communication

A 6-bp deletion in exon 8 and two mutations in introns of \( \text{TYRP1} \) are associated with blond coat color in Liangshan pigs

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Abstract

Melanocortin receptor 1 (MC1R), Agouti signaling protein (ASIP), and Tyrosinase-related protein 1 (TYRP1) are reported critical genes that regulate pheomelanin and eumelanin synthesis in mammals. Liangshan pig is a special Chinese indigenous pig breed with two completely different coat colors, solid black and blond. In this study, we detected polymorphisms of the above three genes and assessed the relationships between the variations and coat color phenotypes in Liangshan pigs. The findings revealed that the blond phenotype of Liangshan pig was related to dominant mutations in \( \text{TYRP1} \), but not related to mutations in \( \text{MC1R} \) or \( \text{ASIP} \). We found three closely linked mutations in \( \text{TYRP1} \), g.8406G→A in intron 4, g.11100A→G in intron 5, and g.17599_17604del in exon 8, that were completely associated with blond coat color in Liangshan pigs. Further analysis revealed that a 6-bp deletion mutation resulted in deletion of Met and Gly residues at positions 495 and 496 in \( \text{TYRP1} \) protein, and altered the structure of transmembrane domain of \( \text{TYRP1} \). Together, our findings indicated that these three mutations in \( \text{TYRP1} \) cause the blond phenotype in Liangshan pigs.

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1. Introduction

Animal coat color is predicted to have three main functions: concealment, communication, and regulation of physiological processes (Caro, 2005; Miyagi and Terai, 2013; Takahashi, 2013). A coat color phenotype depends upon the quality and ratio of eumelanin (black to brown) and pheomelanin (yellow to reddish brown), which are produced by skin-resident melanocytes (Ito and Wakamatsu, 2008; Yang et al., 2015). To date, more than 350 genes involved in coat color have been identified in mammals (Chandramohan et al., 2013), such as the family 7 member 11 gene (Sct7a11) (Tian et al., 2015), Kit ligand (KITLG) (Guenther et al., 2014), and melanophilin (MLPH) gene (Li et al., 2014). Among these genes, Melanocortin receptor 1 (MC1R), Agouti signaling protein (ASIP), and Tyrosinase-related protein 1 (TYRP1) are three major genes that regulate coat color phenotypes in mammals (Chandramohan et al., 2013; Hanna et al., 2014; Kodama et al., 2015; Turnova et al., 2015). Activation of MC1R signaling generates dominant black coloring, whereas loss-of-function mutations are related to a recessive red coat color (Li et al., 2010). Various MC1R mutations associated with coat color have been documented in many mammals, including sheep (Deng et al., 2009), cattle (Russo et al., 2009), horses (Wagner and Reissmann, 2000), dogs (Han et al., 2012), and pigs (Kijas et al., 1998). ASIP, a high-affinity antagonist of MC1R that acts by abrogating the action of a-MSH (Lamoreux et al., 2010), can also influence the production of melanin in many animals, such as dogs (Lightner, 2009), alpacas (Feeley et al., 2011), and sheep (Norris and Whan, 2008). TYRP1 can not only act in concert with dopachrome topoisomerase (DCT) to convert dopaquinone, but also serve to stabilize tyrosinase (TYR) (Kobayashi et al., 1998; Hoekstra and Nachman, 2003). Furthermore, mutations in \( \text{TYRP1} \) have been associated with coat color in many domesticated mammals (Bell et al., 1995; Berryere et al., 2003; Lyons et al., 2005).

Liangshan pig is a famous indigenous pig breed in China. This breed is distributed in Sichuan province and lives in mountainous areas (at about 1500–2500 m altitude) (Shen et al., 2015). Most pigs of this breed are characterized by a solid black coat color, while a few Liangshan pigs have a blond coat color. This study aimed to investigate genetic variability in \( \text{MC1R} \), \( \text{ASIP} \), and \( \text{TYRP1} \) and to assess possible associations between genetic variants and blond coat color in this breed.
2. Materials and methods

2.1. Sampling

A total of 110 genetically unrelated Liangshan pigs, including 58 blond pigs and 52 pigs with a solid black coat color, were sampled from a Liangshan pig nucleus farm in Mabian County, Sichuan Province, China. Ear tissues were collected from each pig and stored in 75% ethanol at 
\[-20 \degrees C]. Genomic DNA was extracted using a DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer’s protocol.

2.2. Primer design and PCR amplification

To identify polymorphisms in \textit{MC1R}, \textit{ASIP}, and \textit{TYRP1}, several pairs of primers were designed according to published sequences (Table 1). Two primer pairs were designed to amplify the entire \textit{MC1R} coding region (AF326520), three pairs of primers were used to amplify the complete \textit{ASIP} coding reading frames (AJ427478), and seven pairs of primers were synthesized to amplify the \textit{TYRP1} exons along with partial intron regions (NC_010443.4). PCR was carried out in a final reaction volume of 25 \(\mu\)L that contained 50 ng DNA, 10 pmol/L each primer, 200 \(\mu\)mol/L each dNTP, 1.5 mmol/L MgCl\(_2\), 3 \(\mu\)L buffer, and 1.0 U TaqDNA polymerase (Takara, Dalian, China). Thermocycling conditions began with a denaturing at 95 \degrees C for 3 min, followed by 35 cycles of denaturing at 95 \degrees C for 30 s, annealing at the Tm (Table 1) for 30 s, and extension at 72 \degrees C for 1 min, and finally one final extension step at 72 \degrees C for 5 min; the procedure was carried out using a PTC-200 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA). PCR products were purified using a QIAEX II Agarose Gel Extraction Kit and were directly sequenced by an ABI 3730XL DNA analyzer (Applied Biosystems, Carlsbad, CA, USA) using the BigDye® Direct Cycle Sequencing Kit.

2.3. Sequence analysis

The identified sequences were characterized using various bioinformatics tools. DNA sequence data were analyzed by DNASTAR 7.1 software. Alignment of \textit{TYRP1} amino acid sequence was performed using ClustalW, while TMpred was utilized to predict the protein domains of \textit{TYRP1}.

2.4. Data analysis

The allele and genotype frequencies of these genes were calculated, and chi square test of the association between phenotypes and genotypes was performed using SAS Software (version 9.2, USA). Haplotype combinations of these three detected polymorphic sites were constructed with the PHASE 2.1.1 software (Stephens and Donnelly, 2003).

3. Results and discussion

3.1. Polymorphism of \textit{MC1R} in Liangshan pigs

To characterize the genetic variation between black and blond coat colors in Liangshan pigs (Fig. 1), we first sequenced the coding regions...
of MC1R gene. Three MC1R alleles were identified, E\textsuperscript{D1} (MC1R*2, MC1R*21, MC1R*23), E\textsuperscript{+} (MC1R*19) and E\textsuperscript{P} (MC1R*6). All these three detected alleles have been documented in Chinese domestic pig breeds in previous report (Li et al., 2010). As shown in Table 2, all of these alleles existed both in black and blond pigs and there are no significant difference of the genotype frequency between black and blond pigs. Considering there were no inactivation mutations have been found in MC1R gene to be responsible for the phenotype of red-colored European breeds, such as Duroc and Leicoma (Kijas et al., 1998; Fang et al., 2009). We concluded that MC1R gene is not responsible for the blond coat color phenotype in Liangshan pigs.

3.2. Variation of ASIP in Liangshan pigs

Ten single nucleotide polymorphisms (SNPs) were identified in ASIP gene, including six synonymous mutations and four non-synonymous mutations (Table 3). All of these SNPs were detected in both solid black and blond pigs. Additionally, most of these mutations have been previously documented in Chinese pig breeds (Mao et al., 2010). Therefore, all of these ASIP mutations were excluded as potential causative mutations for the blond coat color in Liangshan pigs. Meanwhile, the c.305C\textsuperscript{N}T mutation was identified for the first time in pigs. We detected one TT homozygote and six heterozygotes in blond Liangshan pigs and one heterozygote in solid black Liangshan pigs, all of other genotyped pigs were CC homozygotes. Although no functional variation in ASIP was identified in Liangshan pigs, considering that regulatory mutations have been reported to cause coat color changes (Drögemüller et al., 2006; Pielberg et al., 2008), it is worthwhile to further research the regulatory region of ASIP gene.

3.3. The relationship between TYRP1 mutations and blond phenotype in Liangshan pigs

Mutations in TYRP1 that yield different coat colors have been documented in many studies (Utzeri et al., 2014; Turnova et al., 2015). Although it has been documented that TYRP1 variants can be

Table 2

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Allele frequency</th>
<th>(\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(E^{D1}E^{D1})</td>
<td>(no./freq)</td>
<td>36/69.23%</td>
</tr>
<tr>
<td>Black</td>
<td>(E^{D1}E^{D2})</td>
<td>(no./freq)</td>
<td>12/23.08%</td>
</tr>
<tr>
<td></td>
<td>(E^{D1}E^{D3})</td>
<td>(no./freq)</td>
<td>4/7.69%</td>
</tr>
<tr>
<td></td>
<td>(E^{D1}E^{+})</td>
<td>(no./freq)</td>
<td>84.62%</td>
</tr>
<tr>
<td></td>
<td>(E^{D1}E^{P})</td>
<td>(no./freq)</td>
<td>11.54%</td>
</tr>
<tr>
<td></td>
<td>(E^{+}E^{+})</td>
<td>(no./freq)</td>
<td>3.84%</td>
</tr>
<tr>
<td></td>
<td>(E^{+}E^{P})</td>
<td>(no./freq)</td>
<td>0.04%</td>
</tr>
<tr>
<td></td>
<td>(E^{D1})</td>
<td>11.54%</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(E^{+})</td>
<td>3.84%</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(E^{P})</td>
<td>0.04%</td>
<td>ns</td>
</tr>
</tbody>
</table>

\(\chi^2\): chi square value. \(\chi^2 = 5.99, P = 0.05, \chi^2 = 9.21, P = 0.01.\) ns indicated no significant difference of MC1R genotypes between black and blond Liangshan pigs.

Table 3

<table>
<thead>
<tr>
<th>Location</th>
<th>Nucleotide position\textsuperscript{a}</th>
<th>CDS position\textsuperscript{b}</th>
<th>Nucleotide polymorphism</th>
<th>Amino acid polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron1</td>
<td>162900</td>
<td></td>
<td>A\textsuperscript{→}G</td>
<td>–</td>
</tr>
<tr>
<td>Exon2</td>
<td>163016</td>
<td>33</td>
<td>A\textsuperscript{→}G</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>163108</td>
<td>125</td>
<td>T\textsuperscript{→}C</td>
<td>Met\textsuperscript{→}Thr</td>
</tr>
<tr>
<td></td>
<td>163140</td>
<td>157</td>
<td>G\textsuperscript{→}A</td>
<td>Val\textsuperscript{→}Met</td>
</tr>
<tr>
<td>Exon3</td>
<td>164454</td>
<td>202</td>
<td>G\textsuperscript{→}A</td>
<td>–</td>
</tr>
<tr>
<td>Exon4</td>
<td>167718</td>
<td>305</td>
<td>C\textsuperscript{→}T</td>
<td>Pro\textsuperscript{→}Lys</td>
</tr>
<tr>
<td>3‘UTR</td>
<td>167875</td>
<td>462</td>
<td>G\textsuperscript{→}A</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>167921</td>
<td>508</td>
<td>C\textsuperscript{→}T</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>168122</td>
<td>709</td>
<td>G\textsuperscript{→}A</td>
<td>–</td>
</tr>
</tbody>
</table>

\textsuperscript{a} indicates that there is no CDS position.
\textsuperscript{b} indicates that there is no change of amino acid. Met, methionine; Thr, threonine; Val, valine; Glu, glutamic acid; Lys, lysine; Pro, proline.
\textsuperscript{c} Numbering refers to GenBank accession number AJ427478.

\textsuperscript{d} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{e} indicates that there is no CDS position.
\textsuperscript{f} indicates that there is no change of amino acid.

\textsuperscript{g} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{h} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{i} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{j} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{k} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{l} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{m} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{n} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{o} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{p} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{q} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{r} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{s} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{t} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{u} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{v} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{w} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{x} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{y} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{z} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{AA} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{BB} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{CC} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{DD} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{EE} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{FF} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{GG} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{HH} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{II} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{JJ} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{KK} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{LL} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{MM} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{NN} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{OO} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{PP} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{QQ} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{RR} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{SS} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{TT} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{UU} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{VV} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{WW} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{XX} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{YY} The adenine of the start codon ATG of the ASIP cDNA sequence.

\textsuperscript{ZZ} The adenine of the start codon ATG of the ASIP cDNA sequence.
responsible for blond hair in dark-skinned Melanesians (Kenny et al., 2012), no previous studies have identified a relationship between TYRP1 variants and blond phenotype in pigs. Using candidate gene approach, we investigated whether the blond coat color of Liangshan pigs was associated with TYRP1. After sequencing 52 black and 58 blond Liangshan pigs, 31 SNPs were found in TYRP1 (Fig. 2A). Among these 31 SNPs, 21 were located in introns and none of which altered the conserved splice sites, while 10 SNPs were found in exons. The SNPs in exons included four synonymous SNPs (g.1623C>T, g.1671C>T, g.1782T>C, and g.1831C>T) and six non-synonymous SNPs [g.22A>T (p.S24T), g.49C>A (p.L17M), g.54T>A (p.F18L), g.1574A>G (p.H143R), g.11311G>A (p.R417K), and g.17599_17604del (p.M495_G496del)]. Among the 31 mutations within TYRP1, 28 substitutions were present in both black and blond Liangshan pigs that were not further analyzed. Notably, all of the three remaining mutations, g.8406G>A, g.11100A>G, and g.17599_17604del, only appeared in blond Liangshan pigs (Fig. 2). Based on these three SNPs, only two haplotypes were detected in the 110 Liangshan pigs. The haplotypes and their frequencies were listed in Table 4. As shown in Table 4, these three loci exhibited close linkage with each other. Further analysis revealed that g.17599_17604del, a 6-bp deletion mutation in exon 8 of TYRP1, caused a two amino acid deletion (p.M495_G496del). Sequence alignment analysis showed that the deletion occurred in a conserved transmembrane domain of the protein (Fig. 3A), and this mutation was predicted to change the structure of the transmembrane domain (Fig. 3B). Intriguingly, the p.Gly496Asp substitution that affects the same transmembrane domain of TYRP1 has been reported in goats (Becker et al., 2015). We found that the del allele was completely absent from all 52 black pigs (Tables 4 and 5), whereas it was present in all 58 blond Liangshan pigs that we genotyped (Table 5). And it has been documented that none of the 685 pigs without blond coat from other 27 diverse pig breeds and wild boars had the del allele (Ren et al., 2011). Therefore, we inferred that the del allele exhibited a dominant mode of inheritance in blond coat phenotype of Liangshan pigs.

4. Conclusion

We established that the g.8406G>A, g.11100A>G, and g.17599_17604del mutations in TYRP1 are associated with blond coat color phenotype in Liangshan pigs and these three mutations show a close genetic linkage with each other. Moreover, these three mutations can be used as diagnostic markers for solid black and blond coat colors in breeding of Liangshan pigs. Our study provides evidence that TYRP1 is related to the pigmentation of pigs and advances our understanding of the molecular basis for color variation in Liangshan pigs. These findings also established a novel genetic testing tool for Liangshan pig breeding in selecting for or against the blond coat color.

Table 4

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>TYRP1 mutation site</th>
<th>Total (no./freq)</th>
<th>Phenotype (no./freq)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g.8406</td>
<td>g.11100</td>
<td>g.17599-17604</td>
</tr>
<tr>
<td>H1</td>
<td>G</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>H2</td>
<td>A</td>
<td>G</td>
<td>del</td>
</tr>
</tbody>
</table>

Fig. 3. Mutation of the TYRP1 g.17599_17604del. (A) Multispecies alignment of the TYRP1 protein sequence around the deletion. The sequences for the alignment were taken from the following accessions: KT_581974 (LSP), XP_006208355.1 (alpaca), NP_001036025.2 (cat), NP_776905.2 (cattle), NP_001181895.1 (dog), NP_001272656.1 (goat), NP_000541.1 (human), NP_112479.1 (mouse), NP_001284424.1 (rabbit), NP_001132495.1 (sheep). The horizontal line indicates the transmembrane domain of the protein. (B) The structures of the transmembrane domain of the protein. The left one is the structure without mutation and the right one is the structure with the del mutation.
Table 5
Genotypes of TYRP1 gene in Liangshan pig.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype( no./freq)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black</td>
<td>H1/H1: 52/100% 0/0%</td>
<td><strong>11.0</strong></td>
</tr>
<tr>
<td></td>
<td>H1/H2: 5/8.62% 53/91.38%</td>
<td></td>
</tr>
<tr>
<td>Blond</td>
<td>0/0%</td>
<td></td>
</tr>
</tbody>
</table>

χ²: χ² chi square value, χ² = 3.81, P = 0.05, χ² = 6.63, P = 0.01.
** The extremely significant level.

Acknowledgments

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