Chinese White Rongchang Pig Does Not Have the Dominant White Allele of KIT but Has the Dominant Black Allele of MC1R

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The mast/stem cell growth factor receptor (KIT) and melanocortin receptor 1 (MC1R) mutations are responsible for coat color phenotypes in domestic pigs. Rongchang is a Chinese indigenous pig breed with a white coat color phenotype. To investigate the genetic variability of the KIT and MC1R genes and their possible association with the coat color phenotype in this breed, a gene duplication and splice mutation of KIT were diagnosed in a sample of 93 unrelated Rongchang animals. The results show that Rongchang pigs have a single copy of KIT without the splice mutation at the first nucleotide of intron 17, indicating that the dominant white allele of KIT is not responsible for their white phenotype. The KIT mRNA coding sequences were also determined in this breed. Three putative amino acid substitutions were found in the KIT gene between Rongchang and Western white pigs, their association with the Rongchang white phenotype remains unknown. For the MC1R gene, Rongchang pigs were demonstrated to have the same dominant black allele (EΔI1) as other Chinese breeds, supporting the previous conclusion that Chinese and Western pigs have independent domestication origin. We also clarified that the Rongchang white phenotype was recessive to nonwhite color phenotypes. Our results provide a good starting point for the identification of the mutations underlying the white coat color in Rongchang pigs.

As characteristics of many pig breeds, coat color phenotypes have been extensively selected since pig domestication. Numerous coat color genes have been reported in mammals (http://albinismdb.med.umn.edu/genes.htm), of which mast/stem cell growth factor receptor (KIT) and melanocortin receptor 1 (MC1R) are 2 major loci responsible for coat color in pigs (reviewed in Andersson and Georges 2004).

KIT plays a crucial role in the survival and migration of neural-crest–derived melanocyte precursors. To date, 6 KIT alleles have been described in pigs: allele *i* is present in wild boar and colored breeds, allele *IΔP* causes a white belt in Hampshire and most likely in other breeds with the belt phenotype, allele *IΔP* is responsible for patches of white color in Pietrain, Landrace, and Large White, and allele *IΔSFP*, or *IΔF* results in a fully dominant white color in Landrace and Large White (Pielberg et al. 2002). The dominant white *I* allele is associated with a gene duplication and a splice mutation of KIT. The KIT duplication is assumed to act as a regulatory mutation, and the phenotypic effect is due to overexpression or dysregulated expression of KIT (Moller et al. 1996; Marklund et al. 1998). The KIT splice mutation occurs at the first nucleotide of intron 17 in 1 *IΔF* or 2 copies (*IΔF*), the mutant transcript lacks exon 17 and expresses a truncated and presumed loss-of-function protein (Marklund et al. 1998; Pielberg et al. 2002).

MC1R is a G-protein–coupled protein that regulates the production of melanin in melanocyte (Kijas et al. 1998). Seven MC1R alleles have been found in pigs: alleles MC1R*1 and MC1R*5 for the wide type color, alleles MC1R*2, MC1R*7, and MC1R*3 for the dominant black, allele MC1R*6 for red or white with black spots, and allele MC1R*4 for the recessive red (reviewed in Andersson 2003).

Rongchang is a Chinese white pig breed that is characterized for its solid white coat color, sometimes also with small black patches around the eyes and ears (Zhang 1986). The purpose of this study was to investigate the variability in KIT and MC1R and their possible association with the white coat color in this breed. Our results provide evidence that the...
Rongchang white phenotype is not caused by the previously described dominant white allele of KIT (I), and the MC1R variant in this breed is identical to the E^{D1} allele for dominant black that was previously documented in Chinese germplasm, supporting the conclusion that Chinese and Western pigs have different domestication origin.

**Materials and Methods**

**KIT Duplication and Splice Mutation Detection**

To detect the KIT duplication and splice mutation, 95 animals (avoiding full or half sibs) were sampled from a nucleus herd of Rongchang pigs in Chongqing Municipal Breeding Pig Farm. Genomic DNA was extracted from ear notches using a standard phenol/chloroform procedure. The diagnostic test for the KIT duplication was performed as described previously (Giuffra et al. 2002). An 175-bp fragment harboring the KIT splice mutation site was amplified according to Marklund et al. (1998). The KIT splice mutation was detected by single-stranded conformation polymorphism analysis using 14% native polyacrylamide gels (bis:bisacrylamide ratio of 37:8:1) with 2 parallel control samples homoygous for the mutation site. The electrophoresis was run at constant voltage of 120 for 14 h at 4 °C with 1× TBE buffer. The fragments were visualized by silver staining.

**KIT cDNA Isolation**

Total RNA was isolated from ear tissues of 2 unrelated Rongchang boars with RNeasy Midi Kit (Qiagen, Germany) according to the supplier’s protocol. KIT-specific primers f: 5’-GGC TCT TGG GGG TCG GTG TTG C-3’ and r: 5’-TCA GAC ATC TTC GTG GAG CAG AGG-3’ were designed to amplify a 3009-bp fragment covering the entire KIT coding region. Reverse transcription amplification was performed with One-Step RT-PCR Kit (Qiagen) following the manufacturer’s recommendation. PCR products were purified with QIAEX II Agarose Gel Extraction Kit (Qiagen) and subsequently sequenced with the respective PCR primers and a set of internal primers using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (version 3.1) and an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled using SeqMan II from DNASTar 5.0 software package (DNASTAR Inc., Madison, WI). Novel coding single nucleotide polymorphisms (cSNPs) were revealed by comparison of the obtained sequences with known KIT sequences.

**MC1R Allele Determination**

Genomic DNA of 4 unrelated Rongchang boars were used for the determination of the MC1R allele. Two primer pairs (1f: 5’-GCT GAG CAC AGG CGA GGT TTG T-3’; 1r: 5’-AGG AAG CAG AGG CTG GAC AC-3’; 2f: 5’-CGC CAA GAA CCG CAA CCT G-3’; 2r: 5’-GTC CAG CGT CCA TAC CTT CGA C-3’) were designed to amplify the entire MC1R coding sequence and partial flanking sequence according to the porcine MC1R sequence (GenBank accession number AF326520). The amplifications were performed on a PTC-200 thermocycler (MJ Research, Watertown, MA) in a total volume of 25 ml containing approximately 50 ng of genomic DNA, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.2 mM dNTPs, 2 units of Taq DNA Polymerase (Sangon, Shanghai, China), and 10 pmol of each primer. PCR profiles were 95 °C for 2 min, followed by 33 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 45 s, and finally 72 °C for 10 min. PCR products were purified and sequenced. The MC1R allele was determined by comparison of the obtained sequence with known MC1R sequences.

**Results and Discussions**

**The Chinese Aboriginal Rongchang White Pig Does Not Carry the KIT Dominant White Allele Present in Western White Pig Breeds**

The 152-bp fragment spanning the KIT duplication breakpoint was not observed in any of the 95 Rongchang animals (data not shown), indicating the presence of a single copy of KIT in this breed. The splice mutation at KIT intron 17 was not found in either of these animals. All of them had the highly conserved GT dinucleotide at the 5’ splice site of intron 17. We therefore concluded that the dominant white allele of KIT is not responsible for the white coat color phenotype in Rongchang pigs.

**Rongchang and Western White Pigs Have Distinct KIT Sequences**

A 3009-bp KIT cDNA was sequenced in this breed that nearly covers the complete coding region except the last 27 bp of exon 21 (GenBank accession numbers DQ288943 and DQ288944). Two distinct KIT sequences, differing by as many as 10 nucleotide substitutions, were revealed between Rongchang and Western white pigs (Table 1). Six KIT cSNPs were identified within Rongchang pigs, and a total of 18 KIT cSNPs were observed among Rongchang and Western white pigs. Three amino acid substitutions (V^{84M}, R^{173K}, and V^{893F}) were found in exons 2, 3, and 19, respectively (Table 1). The V^{84M} substitution is of particular interest because the substitution was predicted to occur in a first Ig-like loop in an extracellular domain using SMART software (http://smart.embl-heidelberg.de/). It is worthwhile to further determine its functional effects and its association with the white coat color of Rongchang pigs.

**Rongchang Pig Has the Dominant Black E^{D1} Allele at the MC1R Locus**

The MC1R sequence was determined in Rongchang pigs (GenBank accession number DQ288945) by sequence analysis of 2 overlapping 884- and 901-bp fragments spanning the entire MC1R coding region. We found that Rongchang pigs had the E^{D1}(MC1R^{*}2) allele for the dominant black color previously documented in Chinese Meishan pigs (Kijas et al. 1998) (data not shown). We recently demonstrated that
4 Chinese-belted breeds (Jinhua, Shanggao, Dongshan, and Ningxiang) also have the $E^{D1}$ allele differing from the $E^{D2}$ allele in the belted Hampshire (Xu et al. 2006). We constructed a phylogenetic tree using known $MC1R$ sequences (GenBank accession numbers DQ288945, DQ118755, DQ118756, DQ118758, YJ038992, YJ365254, YJ365249–AY365255), Chinese indigenous pig breeds consisting of Rongchang, Meishan and the 4 belt breeds were classified into a clade, which is divergent from the Western breeds (data not shown). Using both mitochondrial DNA and 3 nuclear genes including $MC1R$, Giuffra et al. (2000) have clearly demonstrated that Chinese and Western pig breeds have independent domestication origin. Our result is consistent with their finding and provides additional evidence supporting the conclusion.

The Inheritance Mode and Presumed Molecular Basis for the Rongchang White Phenotype

To clarify the mode of inheritance for the Rongchang white phenotype, 4 Rongchang sows were mated to 1 Large White (dominant white) and 1 Duroc (recessive red) boar. As expected, solid white F1 animals were observed from the cross between Rongchang and Large White. Unexpectedly, offspring of Rongchang × Duroc had solid black coat color with white front legs (data not shown), indicating that the inheritance of the white coat color in Rongchang pigs is recessive to the nonwhite color and the red color is recessive to the black color. The recessive inheritance of the white phenotype is also historically documented in a cross between Rongchang and a Chinese black breed (Neijiang) (Zhang 1986). In this study, we have excluded the dominant white allele of $KIT$ as the molecular basis for the white coat color in Rongchang pigs. Also, we found the presence of the $E^{D1}$ allele of $MC1R$ for dominant black color in this breed. Our explanation for the recessive inheritance of the white color is that it may be caused by loss-of-function or regulatory mutations in genes affecting melanocyte migration/survival, for instance, a mutation similar but not same to the Bel mutation (Giuffra et al. 1999). Offspring from Rongchang × Duroc are expected to inherit normal alleles, such as the $i$ allele, for melanocyte migration/survival from Duroc sire and alleles, such as the $E^{D1}$ allele, for the melanin production from Rongchang sow. Due to the complementary effect, melanocyte precursors can be migrated normally and black eumelanin can be produced in these heterozygous animals, resulting in black coat color phenotype. An alternative explanation for the molecular basis for the recessive white color in Rongchang pigs is that it may be associated with mutations in genes determining melanin production, such as albinism-related genes (http://albinismdb.med.umn.edu/).

An evidence supporting this hypothesis is that Rongchang animals having solid white color without any black patch across the body are always deaf (Zeng DQ, personal communication). In human, at least 21 entries are cataloged in Mendelian Inheritance In Man (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) that have been shown to cause various forms of albinism associated with congenital deafness. However, coat color phenotypes have a complicated genetic basis;

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**Table 1.** Putative nucleotide and amino acid substitutions among 5 $KIT$ cDNA sequences identified in Rongchang and dominant white pigs

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequence variant</th>
<th>KIT*RC1</th>
<th>KIT*0201</th>
<th>KIT*0202</th>
<th>KIT1*0201</th>
<th>KIT1*0202</th>
<th>KIT2*02</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2 (84/3)</td>
<td>C</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>3 (135/4)</td>
<td>T</td>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>17</td>
<td>Present ATG CGC GTT GCA AGA AAT CTC CTC TCT AAC GGA GAG CTT ACG GAT CCC AGT GCG</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
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</tr>
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</table>

**Note:** The EMBL accession numbers for $KIT*RC1$, $KIT*0201$, $KIT*0202$, and $KIT1*0201$, $KIT1*0202$, $KIT2*02$ for each polymorphism is indicated. A dash indicates identity to the master sequence.
other possibility can not be ruled out and further studies are required to test these hypothesis. In conclusion, our study provides a good starting point for the identification of the causative mutations for the recessive white color in Rongchang pigs that will enhance our understanding of mammalian physiology.

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References


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