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Differential expression of CYB5A in Chinese and European pig breeds due to genetic variations in the promoter region

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Summary

Cytochrome b5 (CYB5A) is an important electron transfer protein with homologues in a number of different organisms. In pigs, CYB5A is related to boar taint because of its role in androstenone biosynthesis. To determine the variety of CYB5A expression in pig breeds, genetic variations in the porcine CYB5A promoter region in both Chinese and European pig breeds were examined. Three single nucleotide polymorphisms (NC_010443.4: g.165901487delG, g.165901767T>C and g.165902078C>T) were identified in the porcine CYB5A promoter region. These SNPs occurred in different frequencies in Chinese and European pigs. Chinese pigs were primarily haplotype B (denoted as delG-C-T: the position of nt 165901487 of the CYB5 gene is a G deletion, nt 165901767 is C and nt 165902078 is T), except for Licha black pigs, which were primarily haplotype A (denoted as G-T-C: nt 165901487 is G, nt 165901767 is T and nt 165902078 is C), similar to European pigs. Quantitative PCR data from liver tissues demonstrated that haplotype B individuals had higher CYB5A expression than did those with haplotype A. This was confirmed by in vitro cell transfection assays, in which haplotype B individuals had higher reporter activity than did those with haplotype A. In silico analysis predicted that Myc-associated zinc-finger protein (MAZ) is a potential transcription factor at position 165901767. Electrophoretic mobility shift assays showed this polymorphism affects the stable binding of transcription factors to the CYB5A promoter, which in turn affects the expression levels of this gene. Therefore, this variation of the porcine CYB5A promoter region may explain the differences in androstenone accumulation between Chinese and European pig breeds and may also prove useful as a genetic marker to distinguish the origin of different pig breeds.

Keywords boar taint, electrophoretic mobility shift assay, gene expression, haplotype, transcription factor

Introduction

Cytochrome b5A (CYB5A) is an important electron transfer hemoprotein that serves as a modifier and is an indispensable component of numerous cytochrome P450 (CYP450)-catalysed reactions. CYB5A is a cofactor of the steroidogenic enzyme 17α-hydroxylase/17,20-lyase (CYP17A1), a key enzyme in the steroidogenic pathway (Ozols 1976; Abe & Sugita 1979; Auchus et al. 1998; Yamazaki et al. 1998; Ogishima et al. 2003). CYB5A plays a significant role in intracorporeal metabolism, participating in a series of oxidation–reduction reactions such as desaturation of fatty acids and deoxidisation of ferrihemoglobin (Ozols 1976; Kok et al. 2010).

Previous studies of porcine CYB5A mainly focused on steroid hormone metabolism related to boar taint. Boar taint produces strong odours and flavours in cooked pork from uncastrated male pigs, which negatively affects the use of male pigs for meat production (Xue et al. 1996). Two main compounds are responsible for boar taint, skatole and androstenone. These two compounds are lipophilic and...
therefore readily accumulate in the adipose tissue, leading to taint (Patterson 1968). The degree of boar taint varies between breeds. Previous studies have reported that Chinese breeds reach puberty earlier and accumulate higher levels of boar taint compounds than do European pigs (Prunier et al. 1987; Xue 1991).

CYB5A is particularly important in the biosynthesis of androstenone (Davis & Squires 1999). Leung et al. (2010) determined testicular gene expression patterns in Yorkshire pigs with high and low levels of boar taint using DNA microarrays and compared their results with previously published work on Duroc and Norwegian Landrace boars (Moe et al. 2007). CYB5A was overexpressed in pigs with high androstenone levels in all three breeds. Studies of genetic variation in CYB5A found no polymorphism in the coding regions and a single SNP (G>T) 8 bp upstream of the ATG start codon. This polymorphism was associated with lower fat androstenone levels (Lin et al. 2005) and was also reported as a possible cause of androstenone accumulation (Zamaratskaia et al. 2008).

On the basis of previous research (Lin et al. 2005; Zamaratskaia et al. 2008), we speculate that differences in androstenone accumulation may be due to variation of CYB5A regulation. However, the regulatory mechanisms of CYB5A in pigs are relatively unstudied. Therefore, the aim of this study was to identify and characterise novel genetic variations in the CYB5A promoter region in different pig breeds and to elucidate molecular mechanisms of CYB5A transcriptional regulation.

### Materials and methods

#### Sampling and DNA extraction

Ear samples were collected from 532 pigs with no common parents or grandparents belonging to 17 different pig groups. These pigs included 11 Chinese breeds, three different types of Chinese wild boars and three European breeds (Yorkshire, Landrace and Duroc) (Table 1). Genomic DNA was extracted from ear samples via phenol–chloroform extraction and ethanol precipitation (Old & Higgs 1983). The quantity and purity of DNA were determined by a NanoDrop 2000 (Thermo Scientific). The samples from Chinese pig breeds covered the six traditional types of Chinese indigenous pigs described by Zhang (1986), and all were from preservation farms in China.

#### Promoter amplification and genetic variation analysis

A pair of primers (F1: 5’-AAGGAGGAGTAAGCAATG-3’ and R1: 5’-GAGATGAGCGGAACAGAAGT-3’) was used to amplify the promoter region of the CYB5A gene using the pig genome sequences (GenBank accession no. NC_010443.4) as a template.

The final 917-bp amplicon was generated in a volume of 20 μl containing 100 ng of genomic DNA, 0.5 μl of 10 pmol/μl of each primer, 2 μl of 10× PCR buffer, 2 μl of 2.5 mM dNTPs and 2.5 U Taq polymerase. PCR was performed under the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, 60°C for 30 s, 72°C for 30 s

### Table 1  Haplotype frequency distribution among breeds.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Genotypic frequency</th>
<th>Haplotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AB</td>
</tr>
<tr>
<td>Chinese local</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shanggao</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Qianbei Black</td>
<td>38</td>
<td>0.026</td>
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<tr>
<td>Neijiang</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>Tibet pig</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>Min pig</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>Licha Black</td>
<td>51</td>
<td>0.412</td>
</tr>
<tr>
<td>Hanjing</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Jinhua</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Jiaxing Black</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Lantang</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Wuzhishan</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Chinese wild boars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dongbei Boar</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Zhejiang Boar</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Jiangxi Boar</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>European</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duroc</td>
<td>24</td>
<td>0.667</td>
</tr>
<tr>
<td>Landrace</td>
<td>37</td>
<td>0.676</td>
</tr>
<tr>
<td>Yorkshire</td>
<td>33</td>
<td>0.939</td>
</tr>
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</table>

for 30 s and a final extension at 72 °C for 7 min using a Mastercycler® gradient (Eppendorf Co.). PCR products were purified and verified via cloning and sequencing. Sequences were aligned for identification of mutations. Three polymorphisms were identified: g.165901487delG, g.165901767T>C, and g.165902078C>T. Specific protocols were set up to genotype each mutation and carry out haplotype analysis (Table 2). The indel at position 165901487 bp was typed via capillary electrophoresis using an ABI3700 (Thermo Fisher Inc.) after amplifying a 125-bp-long fragment and restricting it with FspBI.

RNA extraction and cDNA synthesis

Liver tissues were collected from six Yorkshire and Jinhua pigs at 1, 30, 60, 90, 120, 150 and 180 days of age, for a total of 84 pigs sampled. Samples were taken immediately after euthanasia, frozen in liquid nitrogen and stored at −80 °C until use. Total RNA was isolated using TRizol (Invitrogen) and an animal total RNA extraction kit (Tiangen) as per the manufacturers’ directions. RNA quality was determined via the 260/280 wavelength ratio and agarose gel electrophoresis. RNA was reverse-transcribed to cDNA using the Improm-II Reverse Transcriptase kit (Promega).

Quantitative PCR and statistical analysis

Quantitative PCR (qPCR) amplification was performed using SYBR Green Universal Mastermix (Tiangen) as per the manufacturer’s recommendation on a CFX96™ Real-Time system (Bio-Rad). The PCR programme was 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a melting curve analysis (65–95 °C) in the last cycle to evaluate amplification specificity. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used for 30 s, 60 °C for 30 s, with a melting curve analysis (65–95 °C) in the last cycle to evaluate amplification specificity. Hypoxanthine guanine phosphoribosyl transferase (HPRT) – an endogenous control (Chen et al. 2007). The primers

<table>
<thead>
<tr>
<th>Method</th>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing temperature</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing</td>
<td>Forward</td>
<td>FAM-TGGGTCAACAGCGACGATGTA</td>
<td>55.6 °C</td>
<td>162 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTCCGTAGAAGCGAGTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>Forward</td>
<td>Biotin-CGCCCTGCCACACAAGGACC</td>
<td>61 °C</td>
<td>77 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGCAGAAGGGGAGTATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFLP</td>
<td>Sequencing</td>
<td>TGGAGTGGCTGGCGA</td>
<td>54 °C</td>
<td>125 bp</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>AATCATCAGGAGGTCTGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGAACCTCCCTCAACGGCACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMSA</td>
<td>CYB5A-wild type</td>
<td>TGAACCTCCCTCAACGGCACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CYB5A-mutant type</td>
<td>TGAACCTCCCTCAACGGCACCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| The motifs of EMSA experiments are underlined. |  |  |  |

construct. The final activities of the constructs were expressed as the mean ± SE. Additionally, plasmids were also transfected into Hela and Cos7 cells to confirm the transient transfection results.

Prediction of transcription factors and electrophoretic mobility shift assay (EMSA)

Prediction of the transcription factor binding sites for the CYB5A promoter region was performed using the Genomatix (http://www.genomatix.de/en/index.html) and TF Search databases (http://www.cbrc.jp/research/db/TSEARCH.html). Potential transcription factors were determined by combining results from transcription factor prediction and transient transfection.

For EMSA, nuclear extracts from the livers of Yorkshire pigs were prepared using a cytosolic protein–nucleoprotein extraction kit (Viagene Biotech) as per the manufacturer’s directions. Oligonucleotide probes with 5’ biotin labelling were commercially synthesised (Sangon) and purified by high-performance liquid chromatography (Table 1). For competition assays, a molar excess of non-biotin-labelled oligonucleotides was used as cold probes. EMSA was performed using a commercial kit (Viagene Biotech). Probes (300 fmol) and nuclear extract (2 μg) were incubated in a mixture containing 1.5 μl of 10× binding buffer (Non-radioactive EMSA Kit, Viagene Biotech) and 1.5 μg of poly d (I-C), with or without 30 pmol of cold probes in a final volume of 15 μl. Samples were resolved on a 6.5% non-denaturing polyacrylamide gel in 0.25× TBE (25 mM Tris–Borate, 0.5 mM EDTA) in a cold-water bath for 1 h at 180 V. The samples in the gel were transferred to a nylon membrane using 0.5× TBE at 390 mA for 30 min. The membrane was cross-linked using UV light for 10 min to immobilise the bound DNA. Chemiluminescent detection and imaging were carried out according to the manufacturer’s instructions.

Results

Genetic variations of the CYB5A promoter

A total of three SNPs were detected in the CYB5A promoter region (KM067157): g.165901487delG, g.165901767T>C and g.165902078C>T. All sequences found in five Chinese pigs were different from the sequences obtained from NCBI. Testing in 532 pigs found two different haplotypes: haplotype A (denoted as G-T-C: in the CYB5A gene the position of nt 165901487 is T, nt 165901767 is C and nt 165902078 is T). All Chinese pigs and wild boars were dominated by haplotype B except for Licha black pigs, which have haplotype A similar to European pigs (Table 1).

CYB5A gene expression patterns in livers of pigs of different ages

Overall, CYB5A expression in both Jinhua and Yorkshire pigs was significantly higher at 1 day than at other ages. Generally, CYB5A mRNA levels were higher in Jinhua pigs than in Yorkshire pigs. At 1, 90, 120 and 150 days of age respectively, CYB5A mRNA levels were 1.59-, 1.66-, 1.44- and 1.43-fold higher in Jinhua than in Yorkshire pigs. There were no significant differences at 30, 60 and 180 days (Fig. 1).

Promoter activities of CYB5A variants in vitro

The constructs containing haplotype A (pGL3-hapA) and haplotype B (pGL3-hapB) showed that both plasmids had higher luciferase responses compared with the pGL3-basic vector. Haplotype B exhibited approximately a sixfold higher luciferase response than haplotype A (Fig. 2).

Haplotype effects on binding affinity of CYB5A promoter transcription factors

There were no differences in transcription factors binding in the alternative alleles of the g.165901487delG and g.165902078C>T. However, MYC-associated zinc-finger protein (MAZ) was predicted to anchor to the 165901757 bp to 165901777 bp promoter region. EMSA confirmed the binding capacity of target DNA fragment to nuclear extracts. As shown in Fig. 3, both wild and mutant probes can bind with nuclear extracts. The wild probe was the T allele-containing probe, and the mutant probe was the C allele-containing probe. However, band intensity was increased when the mutant probe was used as a target, suggesting the mutant nucleotide at position 165901767 bp improved the binding of nuclear extracts. In competition assays, when the nuclear extracts were incubated in the presence of 100-fold unlabelled oligonucleotides, the protein binding to the labelled probe was undetected, shown by the absence of protein–DNA complexes in the gel shift assay.

Discussion

CYB5A is positively correlated with the production of androstenone in pigs (Davis & Squires 1999), and genetic variation in the CYB5A gene could be a marker to select for low-androstenone pig breeds. Previous studies reported genetic variation of porcine CYB5A in European pig breeds, which was associated with androstenone levels in the adipose tissue (Lin et al. 2005). However, this is the first study examining CYB5A variations in Chinese pig breeds. Genetic variation of CYB5A was detected in both Chinese and European pig breeds. No sequence variation was observed in the amino acid-coding portion of the CYB5A.

gene, but one polymorphism was detected 8 bp upstream of the start codon, consistent with previous findings (Lin et al. 2005). Moreover, the reported polymorphism (c.-8G>T) was examined in 17 different pig populations composed of 949 unrelated pigs using allele-specific PCR. There were no significant differences in SNP distributions between Chinese and European pig breeds (data not shown).

We subsequently extended the sequencing region to 917 bp upstream of the start codon to investigate the genetic variation of the regulatory region of the CYB5A gene among different pig populations. Two different haplotypes, A (G-T-C) and B (delG-C-T), were identified among different pig breeds. Interestingly, haplotype A was the dominant haplotype in Chinese Licha black pigs, which is similar to European pigs but different from other Chinese breeds. This may be the result of recent hybridisation of Licha black pigs with Landrace pigs (Liu 2001). Based on these results, the promoter region of the porcine CYB5A gene is a useful genetic marker for determining the origin of pig breeds, and haplotypes A (G-T-C) and B (delG-C-T) can be used to identify most European and Chinese pig breeds.

Despite the different distribution of haplotypes in pig breeds, the effect of these haplotypes on CYB5A expression remains unknown. Promoter region SNPs have been reported to alter gene expression in both prokaryotes and eukaryotes (Kang et al. 2011) and could change the binding affinity of the promoter to transcription factors (Kim et al. 2013), which are essential for protein expression (Nagore et al. 2013). The pGL3-hapB construct showed an approximately sixfold increase in reporter activity, similar to the qPCR results from liver samples. However, a higher reporter activity induction ratio was found in vitro. This may be due to differences between the transregulation of promoters in vivo/in vitro methods and/or other sequences modulating the transcriptional activity of the proximal promoter in vivo. Transcription factor binding site analyses predicted one putative transcription factor, MAZ, at position 165901767 bp as a possible regulator of CYB5A transcription. In particular, the putative transcription factor has a higher binding affinity for the 165901767 C than for the 165901767 T allele. MAZ is a transcription factor known as SAF-1 (serum accelerator factor-1) in pigs. It is a member of a family of transcription factors containing six Cys2-His2-type zinc fingers. The typical MAZ DNA-binding element is a CT box sequence (CCCTCCC). MAZ has been shown to function as a transcriptional activator (Bossone

Figure 1 Expression of CYB5A in livers of Yorkshire and Jinhua pigs. *P < 0.05.

Figure 2 Analysis of CYB5A promoter activity with different haplotypes. The Y-bar shows the two constructs and empty vector (pGL3-basic). The X-bar shows the luciferase activity after normalisation to the cotransfected reference vector (pRL-TK) relative to the activity of the pGL3-basic vector. Data are expressed as the mean and SE of three replicates. hapA, G-T-C (NC_010443.4: g.165901487insG, g.165901767C>T); hapB, delG-C-T (NC_010443.4:g.165901487delG, g.165901767T>C and g.165902078C>T). HepG2, Hela and Cos7 are three stable cell lines.

indicated that the polymorphism at g.165901767T position promotes the transcriptional activation of the gene. Put together, the results of the EMSA assay indicates that the DNA protein complex binding to the variant DNA sequence allele C rather than to the wild-type sequence. Given the enhanced expression that the C variant has shown both in vivo and in vitro over haplotypes containing the T variant, this indicates that the DNA–protein complex binding to the 165901767 position promotes the transcriptional activation of the gene. Put together, the results of the EMSA assay indicated that the polymorphism at g.165901767T>C may be involved in regulation of CYB5A expression, as the intensity of the DNA–protein complex was increased in the presence of the g.165901767-allele C.

Our results showed differential expression of CYB5A gene between Chinese and European pig breeds, which is due to genetic variations in the CYB5A promoter region. The level of CYB5A gene expression was higher in Chinese breeds than in European breeds at the same age. Chinese pig breeds reach sexual maturity much earlier than do European breeds (Xue 1991), and development of early puberty may require higher CYB5A gene expression, as this encodes a key enzyme in steroid biosynthesis. The high androstenone accumulation of Chinese pig breeds has been widely reported (Prunier et al. 1987), and the CYB5A gene was overexpressed in pigs with high androstenone levels (Leung et al. 2010). The average fat androstenone level is 2.80 μg/g in Chinese and 1.4 μg/g in European pigs (Zeng, 2002; Zamaratskaia et al. 2005). Therefore, we speculate that the androstenone accumulation variation between Chinese and European breeds might be associated with variable expression of CYB5A. This may be due to specific genetic variations in CYB5A in the promoter region.

Overall, this study provides a detailed analysis of the regulatory region of porcine CYB5A gene among different breeds. We identified two haplotypes in the promoter region of CYB5A. European pig breeds and Licha black pig breeds were dominated by haplotype A (G-T-C), whereas other groups of Chinese breeds and wild boars were dominated by haplotype B (delG-C-T). Haplotype B in the CYB5A promoter region caused an increase in the level of CYB5A gene expression, likely due to increased binding of transcription factors, which induced transcription in the region containing the g.165901767T>C variant.

Acknowledgements

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