Expression of HMGR and corresponding cholesterol content in tissues of two pig breeds

G.-F. Liu1,2a, W.-N. Fang1, H.-C. Lin1,3, X.-F. Wang4, J.-L. Fu1,2* and A.-G. Wang1,2*

1College of Animal Science and Technology, China Agricultural University, Beijing, 100193, China; 2Key Laboratory of Animal Genetics and Breeding, Ministry of Agriculture, Beijing, 100193, China; 3Institute of Animal Science and Veterinary Medicine, Shandong Academy of Agricultural Science, Jinan, 250100, China; 4Animal Husbandry & Veterinary Service Station, Beijing, 100107, China

(Received 5 November 2007; Accepted 28 April 2009; First published online 29 May 2009)

The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is an essential enzyme in cholesterol biosynthesis. To study the expression of HMGR and corresponding cholesterol content in liver, adipose and muscle, six Chinese local breed (Huai pig) and Landrace pigs were selected. The results indicated that significant differences of cholesterol content in adipose (P < 0.01), liver (P < 0.05) and muscle (P < 0.01) tissues were detected between pigs of differing genetic backgrounds. HMGR mRNA expression were noted for adipose, liver and muscle of the two vastly differing genetics. Moreover cholesterol content differed (P < 0.01) among tissues across breed. Likewise, HMGR mRNA expression was different between adipose and liver tissues, muscle and liver tissues in both breeds; however, no difference was noted between adipose and muscle tissues. Results from this study indicate that cholesterol content and HMGR mRNA expression are higher in Huai pig tissues suggesting this gene is expressed in a breed- and tissue-dependent manner in pigs. Understanding the causes of variation in HMGR gene expression may provide crucial information about cholesterol biosynthesis.

Keywords: 3-hydroxy-3-methylglutaryl-CoA reductase, cholesterol, mRNA expression, pig

Introduction

Cholesterol is a fat-like substance in the body. Despite cholesterol’s bad reputation, the body needs cholesterol to maintain cell membrane functionality, synthesis of steroid hormones and production of bile acids, which aid in fat absorption in the intestines. Moreover, cholesterol is a major component of the myelin sheaths and therefore is essential for optimal neuronal health (Tabas, 2002). As a result, the body needs cholesterol, which can be derived from dietary sources or synthesized by the liver. Myant (1990) has previously reported that precise control of plasma cholesterol levels is critical because excessive cholesterol is known to predispose to the development of atherosclerosis and cardiovascular disease.

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonate and is considered as the rate-limiting enzyme in the overall pathway of cholesterol biosynthesis (Istvan and Deisenhofer, 2000; Jiang et al., 2006). Leveille et al. (1975) showed that liver is an important site of cholesterol synthesis and is controlled by gene of HMGR. Moreover, liver is also the only site in which substantial amounts of cholesterol are removed from the body by excretion into bile acids. However, Sato et al. (2003) found that lipogenic activity in chicken and human liver is much greater than that in adipose tissue, whereas in rat and swine, adipose tissue is the major site of cholesterol synthesis.

The objective of the present study was to evaluate the effects of breed and tissue on cholesterol content and HMGR expression, and analyzed the reason of cholesterol content difference between Chinese local breed of pig (Huai pig) and exotic breed (Landrace).

Material and methods

Animals and tissue collection

Six Huai and Landrace pigs each were individually housed at the Fujian Shanghang Huai pig breeding farm under same feeding and pen conditions. Pigs were slaughtered at saleable body weight (Landrace: 100 kg; Huai pig: 70 kg) by commercially acceptable practices. Within 20 min of exsanguination,
approximately 50 to 100 mg samples were collected from the liver, muscle (Longissimus dorsi), and adipose tissues (back-fat). Samples were snap-frozen in liquid nitrogen, and stored at −80°C for later mRNA extraction.

Molecular cloning of pig HMGR
General methods. Our research groups had obtained the complete pig HMGR CDNA (GenBank accession No. DQ432054) by reverse transcriptase-PCR (RT-PCR) and 3'and 5'-RACE (rapid amplification of cDNA ends) (Wang, 2006).

RNA extraction. Total RNA was extracted by TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD, USA) in accordance with manufacturer’s instructions, and then stored at −80°C. The purity and integrity of RNA was evaluated using electrophoresis and ethidium bromide staining and by optical density (OD) absorption ratio OD260/OD280 and rRNA (28S/18S) ratios. Total RNA (2 μg) was reverse-transcribed in the presence of polythymidine oligonucleotide primers (Oligo-dT18) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT; Promega Corporation, Madison, WI, USA) in 20 μl of reaction volume.

Quantification of genes mRNA using real-time quantitative RT-PCR
PCR amplification. The primers of HMGR and glycerol-aldehyd-3-phosphat-dehydrogenase (GAPDH) genes were designed through using the Beacon Designer 2.1 software. Primers were synthesized in Beijing Ubiogene Science and Technology Corporation (Beijing, China). Sequences of primers and annealing temperature are shown in Table 1.

To validate primers, PCR was performed using 1.5 μl cDNA template, 2.5 μl 10X PCR buffer (containing 100 mM Tris-HCl (pH 8.0), 500 mM KCl, 10 mM of MgCl2 and 0.1% glutin), 2.0 μl 10 mM dNTPs mix, 0.6 μl forward and reverse primers (10 pmol/μl), 0.5 μl AmpliTaq DNA polymerase (5 U/μl) and 17.3 μl double-distilled water (the reagents all came from the National Laboratories for Agro-biotechnology, China Agricultural University, Beijing, China). Amplifying conditions of PCR were 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, annealing for 30 s, 72°C for 1 min. The PCR products were observed on 1.5% agarose gels, visualized by ethidium bromide staining and analyzed using an Alpha Innotech (San Leandro, CA, USA) imaging system.

Once validated, multiplex PCR was carried out in 96-well plates through using 1 μl cDNA template. A 15 μl reaction sample contained 7.5 μl SYBR Green PCR Master Mix (Applied Biosystems Incorporation, Foster City, CA, USA), 0.5 μl forward and reverse primers (10 pmol/μl) and 5.5 μl double-distilled water. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed with 40 cycles at 95°C for 30 s and 60°C for 1 min. Data were collected using the ABI 7900HT Sequence Detection System (Applied Biosystems). Samples were normalized using the housekeeping gene GAPDH (Table 1). All real-time PCR reactions were established in duplicate for target gene and standard curve were established in duplicate for each gene. Pooled liver cDNA was used to create a standard curve for quantification of the transcript using the relative standard curve method as described by Applied Biosystems (Use Bulletin, 1997). Standard curve arbitrary units were set at one for pooled samples and six gradient dilutions were then performed. For each experimental sample, the amount of target gene mRNA relative to GAPDH was determined from their respective standard curves. Relative quantity ratios were obtained by dividing the relative quantity units of target genes by those of GAPDH. Mean values from duplicates were then used to perform statistical analyses.

Relative quantification of genes expression. Quantification of transcripts was performed by comparative C T method and standard curve method (Fink et al., 1998; Favy et al., 2000; Lord et al., 2006). Standard curve values were used for statistical analyses.

The C T method consists of the normalization of the number of target gene copies to an endogenous reference gene (i.e. GAPDH) and is designated as the calibrator, in order to normalize quantity and quality of the cDNA samples.

Relative quantification is given by the following equation:

$$\frac{R}{T} = K(1 + E)^{(C_T,R - C_T,T)},$$

where T is the initial number of target gene copies, R, initial number of standard gene copies, E, efficiency of amplification, C T,R, threshold cycle of target gene, C T,T, threshold cycle of standard gene and K, constant.

In general, the efficiency of PCR reaction was close to one (Use Bulletin, 1997). Although the exact value of K does not be equal to one, the affecting factors will vary only negligibly among single samples so that K is assumed to be equal. So the following equation is given:

$$\text{Mean} \left( \frac{T}{R} = 2^{-\Delta C_T} \right).$$

The second method was performed according to the standard curve described by Applied Biosystems.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of oligonucleotide primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Names</strong></td>
<td><strong>Forward primers</strong></td>
</tr>
<tr>
<td>HMGR</td>
<td>CATCCACCCAAGGTTGTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTCCACTGGTGCTTCACGA</td>
</tr>
</tbody>
</table>
**Cholesterol level detection.** High performance liquid chromatography (HPLC) method was applied to detect the cholesterol concentration in tissues. Liver (0.15 g), adipose (0.3 g) and muscle (1 g) tissues were respectively put in Pyrex test tube; internal standard liquid (1 ml) and saponified liquid (7.3 ml) were added into it. Air above the liquor was removed by overflowing nitrogen gas and placed the test tube in 80°C water-bath until the solution clarified completely. Then the tubes were placed on ice to chill and directly extracted with petroleum ether. The supernatant liquor was taken out and put in a clean test tube. The appropriate volume liquor was collected by drying and concentrating and then analyzed in capillary chromatography. The recovery of this method was between 92.58% and 104.68%. The satisfied results can be obtained under these chromatographic conditions, and cholesterol level determination can be finished within 12 min for each sample (Fang Wenning, unpublished data).

**Statistical analysis**

The data when comparing two breeds were determined using t-test. The other data were analyzed by ANOVA. Significant differences were detected by Duncan’s multiple range tests using GLM procedure of SAS software (version 8.02; SAS Institute, Inc., Cary, NC, USA). Results were presented as means of mRNA relative abundance and cholesterol content ± s.e. Statistical significance was set at $P < 0.05$ and $P < 0.01$.

**Results**

**Cloning and sequence analysis of porcine HMGR cDNA**

The pig HMGR gene contained 20 exons and 19 introns. This gene was assigned to pig chromosome 2q21-22 by RH (radiation hybrid) mapping. The length of its cDNA is 2864 bp (GenBank accession No. DQ432054), including an open reading frame covering 2658 bp. The homology analysis by BLAST software (Basic Local Alignment Search Tool) showed that the identity of pig HMGR and human HMGR was very high (91%; Wang, 2006).

**Real-time quantitative PCR**

The housekeeping gene, GAPDH, was used as the control to show the normalization of the templates in reactions, and standard curves were prepared through using highly concentrated cDNA and gradient dilution, and was used when $R$ was above 0.997.

**Cholesterol content analysis**

**Breed effects.** Cholesterol concentrations were higher in Huai pigs as compared to Landrace in all tissues (Figure 1): adipose tissue ($P < 0.01$), liver ($P < 0.05$) and muscle ($P < 0.01$).

**Tissues effects.** Within both breeds, cholesterol concentrations were higher in liver, intermediate in adipose tissue and lowest in muscle (Figure 2). Landrace’s result is showed in Figure 2a and Huai pig’s result is showed in Figure 2b.

**Figure 1** The cholesterol concentration difference in three tissues adipose (a), liver (b), muscle (c) of two breeds (Landrace and Huai pig). Each bar represents mean ± s.e. *$P < 0.05$; **$P < 0.01$.

**Gene expression analysis**

**Differences in mRNA abundance of HMGR in three tissues across breed.** Using real-time PCR, porcine HMGR mRNA levels were quantified in three tissues between two different kinds of breeds. Results showed that there were differences in HMGR expression in adipose tissue (Figure 3a; $P < 0.05$), liver tissue (Figure 3b; $P < 0.05$) and muscle tissue (Figure 3c; $P < 0.01$) between two breeds.

**Differences of mRNA abundance of HMGR in three tissues of each breed.** Porcine HMGR mRNA levels were quantified in three tissues of each breed. The results showed that the HMGR mRNA level in liver in Landrace was higher than in adipose (Figure 4a; $P < 0.01$) and muscle (Figure 4a; $P < 0.01$), and the difference between the adipose and muscle was not significant (Figure 4a; $P > 0.05$). The trend in Huai pig was the same as in Landrace (see Figure 4b).

**Pearson Correlation Coefficients between HMGR gene and cholesterol.** We compared the correlation between cholesterol concentration and gene expression of HMGR.
The result showed that the Pearson Correlation Coefficients between HMGR gene and cholesterol was 0.46 ($P < 0.01$).

**Discussion**

Huai pig is considered a highly desirable Chinese local breed because it is a miniature breed and produces high quality pork. However, Huai pig is a lard-type breed and the fat content of Huai pigs is considerably higher than that of Landrace pigs. The present research showed that cholesterol content was higher in liver, adipose and muscle from Huai pig than in the corresponding tissues from Landrace pigs. Harris et al. (2004) reported genetic selection for high or low plasma cholesterol is not a good selection indicator of cholesterol or fat accretion, yet argued that the source of dietary fat may influence total fat content in muscle tissues. According to Dorado et al. (1999), there is a high correlation between cholesterol content and fat content ($r = 0.88$, $P < 0.05$). Thus, the difference in cholesterol content between the two breeds may be related to overall fat content.

The HMGR gene has been widely cloned in many species, i.e. the human HMG-CoA reductase gene has been localized to human chromosome 5q12 by *in situ* hybridization and contains 20 exons and 19 introns (Humphries *et al.*, 1985; Luskey and Stevens, 1985), chicken (Sato *et al.*, 2003), rat (Simonet and Ness, 1988; Sundaresan *et al.*, 1989; Duckworth *et al.*, 1991) and some plants (Ha *et al.*, 2003; Jiang *et al.*, 2006). Information regarding this gene in pigs, however, is limiting. In recent years, our group reported firstly, the HMGR gene and the sequence information of HMGR in pig. The research found that nucleotide acid (91%) and amino acid sequence (95%) of pig HMGR were very similar to human HMGR gene (Wang, 2006).

In this study, Huai pig HMGR mRNA level was lower than the corresponding tissues of Landrace in muscle and adipose tissues. However, the cholesterol content in adipose and muscle tissues of Huai pig was higher than the corresponding tissues of Landrace. Cholesterol content as a biological trait was affected by many factors. Besides the level of HMGR mRNA expression, the cholesterol content was affected by other levels such as the processing and modifying of transcription and translation levels. In addition, the cholesterol 7 alpha-hydroxylase (CYP7A1) is an essential enzyme of cholesterol excretion. The substantial amounts of cholesterol are removed from the body by this
enzyme. Because the role of this enzyme was different in different tissues, it led to different cholesterol content in different tissues. These factors may affect the amount of cholesterol in tissues. At present, there was no research of CYP7A1 in pig, so a further research of this enzyme would be done.

In pigs, HMGR mRNA level was high in most tissues, as measured by RT-PCR and Northern blot analysis, however, the highest levels were found in liver, brain and ileum (Wang, 2006). The tissue expression pattern showed that HMGR had strongly expressed differences, which suggested that HMGR might be a constitutively expressing gene. The similar results were reported by Jiang et al. (2006). In addition, the expression pattern in different tissues suggested that this gene is expressed in a tissue-dependent manner in pigs. Our results also show that HMGR expression in liver is higher than that in the adipose and muscle. This suggested that liver is the major site of the cholesterol synthesis and it is possible to decrease the cholesterol content by controlling HMGR expression.

HMGR is the essential enzyme in cholesterol synthesis pathway. Initially, physicians were skeptical of a causal link between cholesterol and coronary heart disease because most patients with the disease had plasma cholesterol only slightly higher than the general population average (The Lipid Research Clinics Coronary Primary Prevention Trial results (LRC-CPTT), 1984; Baum et al., 1998; Tobert, 2003). Furthermore, Ness (2003) reported that supplementing animal diets with cholesterol can lower hepatic HMGR activity. The efficacy of clinically inhibiting HMGR by various statins and reducing cardiovascular morbidity and mortality has been convincingly demonstrated in primary and secondary prevention trials (Scandinavian Simvastatin Survival Study, 1994; Shepherd et al., 1995; Rasmusseen et al., 2001). Recently, HMGR inhibitors have been applied as therapy for the diseases caused by the high level of cholesterol in human; the clinical effect has won widely approval.

Because the similarities between humans and pigs also exist in renal function, vascular structure and respiratory rates, pigs are used as model organism in many areas of medical research including obesity, cardiovascular disease, diabetes, nephropathy and organ transplantation (Dodds, 1982; Lee et al., 1986). The evidence points out that the pig is accepted as an appropriate animal model to access the relationship between plasma cholesterol concentration and coronary artery disease, because of its similarities to human (Pond et al., 1986; Stanton and Mersmann, 1986; Van et al., 1991). Due to the pressing research for human disease, the small-size pigs have been deemed to ideal subscriber. Thus, the Huai pig may serve as an ideal research model for studying this malady, as it is small and easy to handle as well.

Acknowledgements

The authors greatly appreciate Fujian Shanghang Huai pig breeding farm for collecting samples. This study was supported by National Scientific and Technical Supporting Programs of China (No. 2006BAD01A08 & 2006BAD13B08).

References


Dodds WJ 1982. The pig model for biomedical research. Federation Proceeding 41, 247–256.


HMGR and cholesterol content in pig


Myant NB 1990. Current approaches to the genetics of coronary heart disease (CHD) including an account of work done at Hammersmith Hospital. Bollettino della Società italiana di biologia sperimentale 66, 1015–1041.


