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Association of the polymorphism in *GYS1* and *ACOX1* genes with meat quality traits in pigs

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Phenotypic information about several pig meat quality traits on 334 Large White × Meishan F_2 pigs was collected. Effects of the association of the FokI *variants in the seventh intron of the skeletal muscle glycogen synthase (*GYS1*) gene and the* PstI *variants in the ninth intron of the palmitoyl acyl-CoA oxidase 1 (*ACOX1*) gene on the meat quality traits were examined on all pigs. The* FokI *variants of the* GYS1 *gene showed significant effects on pH of* m. semipinalis capitis *(*$P < 0.05$*). Linkage analysis indicated that the peak of the quantitative trait loci (QTL) curve was located around this marker for pH, but it did not reach significance (*$P > 0.05$*). The results may be due to several reasons such as linkage disequilibrium to the causal mutations, the limited number of animals or balance of another QTL or marker with negative effects. Significant effects of* PstI *variants of* ACOX1 *gene were also found on meat colour value and meat marbling score of both* m. longissimus dorsi *and* m. biceps femoris *(*$P < 0.05$*). Dominant effects for the affected traits at those two loci were significant except for meat marbling score of* m. biceps femoris *(*$P < 0.05$*). The results of this study give us some evidence for the potential of those dominant markers used in the marker-assisted selection of crossbreeding of the Large White pig sire lines and Meishan-derived synthetic dam lines.*

Keywords: *ACOX1*, *GYS1*, marker-assisted selection, meat quality, pigs

Introduction

For the last years, meat quality traits have increasingly attracted more attention in pig breeding because selection for high growth rate and lean meat deposition resulted in reduction of meat quality such as the increasing incidence of PSE (pale, soft and exudative) muscle and decreasing of intramuscular fat (IMF) content (Zuo *et al*., 2003). Use of marker-assisted selection (MAS) is especially interesting for meat quality traits, because improvement of meat quality is difficult using conventional selection methods since most traits of interest can only be measured after slaughter and, therefore, only information on relatives can be used for selection (Malek *et al*., 2001). The successful application of MAS in the animal population will depend on the identification of major genes or tightly linked markers. The study of associations of candidate genes is a step for the knowledge of the genetic basis of productive traits, and compared with other genomic approaches (quantitative trait loci (QTL) detection) is potentially more easily and efficiently implemented in breeding programmes (Ovilo *et al*., 2006).

The skeletal muscle glycogen synthase (*GYS1*) gene is a key enzyme of non-oxidative pathway of glucose metabolism that has been reported to be related to insulin resistance in non-insulin-dependent diabetic (NIDDM) patients (Rissanen *et al*., 1997; Shimomura *et al*., 1997; Orho-Melander *et al*., 1999; Fenger *et al*., 2000; Motoyama *et al*., 2003). The A/G mutation in intron 14 of the porcine *GYS1* gene has been discovered, but has no effect on the content of glycogen in skeletal muscle (Te Pas *et al*., 2003). Pre-oxisomal acyl-CoA oxidase 1 (ACOX1) catalyses the beta-oxidation of very long-chain fatty acids, and thus plays an essential role in fatty acid degradation. Fan *et al*. (1996) found that homozygous *ACOX1*-null mice were viable, but growth retarded and infertile. Expression of the *ACOX1* gene was significantly increased in male mice fed a high-fat diet, compared with a low-fat diet (Kim *et al*., 2004). The porcine *GYS1* and *ACOX1* gene was physically mapped to SSC6 and SSC12, respectively (Cirera *et al*., 2003; Fontanesi *et al*., 2003), where significant QTL affecting meat quality traits have been reported (De Koning *et al*., 1999; Clop

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et al., 2003). Therefore, those two genes have been suggested as the promising candidate genes for meat quality traits, given their roles in the glycogen synthesis and metabolism of fatty acids.

Compared with the Chinese fat-type Meishan pigs, the meat-type Large White pigs have a higher growth rate, higher feed to body weight conversion ratio and higher carcass lean percentage, but have lower IMF and inferior eating quality from the Chinese perspective. In order to seek for the major gene or marker underlying the important economic traits such as meat quality traits, a three generation resource family was established by Large White boars and Meishan dams in our lab. The objective of the current study was to detect *Fok*I restriction endonuclease variants in the seventh intron of the *GYS1* gene and *Pst*I restriction endonuclease variants in the ninth intron of the *ACOX1* gene in the Large White × Meishan pig resource family, and to determine whether these were associated with variation in meat quality traits, and thus could contribute to breeding programmes.

Material and methods

Animals and data collection

Two F_2 populations used in the association analysis were derived from the intercross of Large White and Meishan pigs. One population was formed by 140 F_2, 28 F_1 and 10 grandparent animals in 16 full-sib families, and the other population consisted of 194 F_2, 26 F_1 and 11 grandparent animals in 21 full-sib families. They were fed twice daily with diets formulated according to age under a standardised feeding regimen and free access to water. The average live weight at slaughter was 87.0 ± 7.07 kg. The F_2 pigs were slaughtered at 2000 and 2003 following a common protocol (Xiong and Deng, 1999). Meat quality traits including pH of *m. longissimus dorsi* at post slaughter 45 min (pHLD), pH of *m. biceps femoris* at post slaughter 45 min (pHBF), pH of *m. semipinalis capitis* at post slaughter 45 min (pHSC), drip loss rate (DLR, %), water-holding capacity (WHC, %), meat colour value of *m. longissimus dorsi* (MCV1), meat colour value of *m. biceps femoris* (MCV2), meat marbling score of *m. longissimus dorsi* (MMS1), meat marbling score of *m. biceps femoris* (MMS2), IMF (%) and water moisture (WM, %) were measured according to the method of Xiong and Deng (1999). Genomic DNA was prepared from blood samples using a standard phenol: chloroform extraction method.

PCR-RFLP genotyping

The DNA from the F_2 pigs was used as template to perform PCR. According to the obtained cDNA sequences (Genbank accession number AY870324) and the exon/intron organisation of human *GYS1* gene, PCR primers (forward: 5′- TAT GAG TTC TCC AAC AAG GGG -3′; reverse: 5′- GAT GAA GAA AGC AAC CAC TGT C -3′) were designed to amplify porcine *GYS1* gene. According to the obtained cDNA sequences (Genbank accession number DQ842227) and the exon/intron organisation of human *ACOX1* gene, PCR primers (forward: 5′- GGA AAT GAA CCC GAC CAG TA -3′; reverse: 5′- TGC GTC TCA GGA AGC AGT AAG -3′) were designed to amplify porcine *ACOX1* gene. The reaction mixtures comprised 25 ng porcine genomic DNA as template, 0.25 μmol/l of each primer, 0.25 μmol/l of each dNTP, 1 × PCR buffer and 1 U *Taq* DNA polymerase (Biostar Internation, Toronto, Canada). The PCR amplifications were performed in 20 μl on a GeneAmp PCR system 9600 (Perkin Elmer, Foster City, CA, USA) with the following cycling parameters: 95°C initial denaturation for 4 min, 35 cycles of 95°C denaturation for 45 s, 60°C (*GYS1*) or 61°C (*ACOX1*) annealing for 45 s, and 72°C extension for 45 s. A final extension was performed at 72°C for 10 min. 8 μl of PCR amplifications obtained with above primer pairs were digested with 10 U *Fok*I and *Pst*I restriction enzyme (TaKaRa, Dalian, China), electrophoresed on 1.5% (*GYS1*) or 2.5% (*ACOX1*) agarose gel in 1 × TAE buffer and stained with 0.5 μg/ml ethidium bromide.

Analysis

The effects of single genotypes on the traits studied were analysed by the least-squares method as applied in the general linear model (GLM) procedure of Statistical Analysis Systems Institute (SAS; 2000) according to the following statistical model:

$$T_{ijkl} = \mu + S_i + Y_j + G_k + F_l + b_{ijkl}X_{ijkl} + e_{ijkl},$$

where T_{ijkl} is the observed values of a given trait, μ is the overall mean, S_i is effect of sex ($i = 1$ for male or 2 for female), Y_j is the effect of year ($j = 1$ for year 2000 or 2 for year 2003), G_k is the effect of genotype ($k = AA$, AB and BB), F_l is the effect of family (l=37), b_{ijkl} is the regression coefficient of the slaughter age for meat quality traits, X_{ijkl} is the slaughter age, and e_{ijkl} is the random residual. Both additive and dominance effects were estimated using the REG procedure of SAS version 8.0, where the contrast coefficients for the additive effect were denoted as −1, 0 and 1 for *AA*, *AB* and *BB*, respectively, and the contrast coefficients for the dominance effect were denoted as 1, −1 and 1 for AA, AB and BB, respectively (Liu, 1998).

In order to determine whether the significant associations of *GYS1* gene were due to the marker or due to other co-inherited blocks, the genetic mapping were performed by CRI-MAP software version 2.4 (Green *et al.*, 1990) using genotypes for *Fok*I PCR-RFLP and four microsatellite markers (*SW1302*, *SW1473*, *S0121* and *SW322*) information available on SSC6 (Zuo *et al.*, 2005; Zhang *et al.*, 2007). Least-square regression interval mapping as described by Haley *et al.* (1994) was used for QTL detection. QTL analysis was carried out on the Internet (http://qtl.cap.ed.ac.uk). For the meat quality traits, sex, year and full-sib family were included as fixed effects with the slaughter date as a covariate.

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Table 1 *Phenotypic means, standard deviation (s.d.) and coefficients of variation (CV) for meat quality traits*

Trait	Symbol	No.	Mean	s.d.	CV (%)
pH (*m. longissimus dorsi*)	pHLD	334	6.34	0.18	2.89
pH (*m. biceps femoris*)	pHBF	334	6.40	0.13	2.03
pH (*m. semipinalis capitis*)	pHSC	334	6.44	0.12	1.86
Drip loss rate	DLR	327	6.25	1.44	23.02
Water-holding capacity	WHC	327	91.50	2.05	2.24
Meat colour value (*m. longissimus dorsi*)	MCV1	334	20.23	3.24	16.01
Meat colour value (*m. biceps femoris*)	MCV2	334	19.05	1.70	8.92
Meat marbling score (*m. longissimus dorsi*)	MMS1	334	3.43	0.24	7.00
Meat marbling score (*m. biceps femoris*)	MMS2	334	4.10	0.18	4.39
Intramuscular fat (*m. longissimus dorsi*)	IMF	334	3.19	0.86	27.00
Water moisture (*m. longissimus dorsi*)	WM	334	73.78	0.77	1.04

Table 2 *Distribution of genotypic and allelic frequencies in the resource population*

				Genotype			Allele frequency	
Gene	Populaton (year)	Generation	No.	*AA*	*AB*	*BB*	*A*	*B*
GYS1	Population (2000)	F_0	10	3	2	5	0.40	0.60
		F_1	28	2	26	0	0.54	0.46
		F_2	138	35	72	31	0.51	0.49
	Population (2003)	F_0	11	3	4	4	0.45	0.55
		F_1	26	9	17	0	0.67	0.33
		F_2	172	67	84	21	0.63	0.37
ACOX1	Population (2000)	F_0	10	3	0	7	0.30	0.70
		F_1	28	0	28	0	0.50	0.50
		F_2	139	35	71	33	0.51	0.49
	Population (2003)	F_0	11	3	0	8	0.27	0.73
		F_1	26	0	26	0	0.50	0.50
		F_2	159	37	88	34	0.51	0.49

Results

Phenotype, genotype and allele frequencies

Phenotypic means, standard deviations (s.d.) and coefficients of variation (CV) for meat quality traits were given in Table 1. From this table, it was found that the CVs ranged from 1.04 to 31.80%. The traits such as DLR, meat colour value, IMF content had the higher CV, while the other traits showed relatively lower variation. The distribution of genotypic and allelic frequencies in the pig population is given in Table 2. Overall, the allele frequencies showed almost equal proportion of alleles for these two genes except the *GYS1* allele frequency in population 2003. For the analysis of the combined genotypes, the expected frequencies of the genotypes and their combinations were calculated by simple allele counting. All of the 9 (3^2) theoretically possible combinations of two individual genotypes were observed and most of the combined genotypes found clearly followed the Hardy–Weinberg equilibrium (Table 3), showing that both genes, *GYS1* and *ACOX1*, are independent which seems reasonable as they are located in different chromosomes, *GYS1* in chromosome 6 and *ACOX1* in chromosome 12.

Table 3 *Expected frequencies of combined genotypes and comparison of observed and expected numbers of animals*

		No. of animals	
Combined genotypes	Expected frequency	Observed	Expected
AAAA (1)	0.0792	28	23.2
AAAB (2)	0.1782	49	52.2
AABB (3)	0.0726	22	21.2
ABAA (4)	0.1200	30	35.2
ABAB (5)	0.2700	83	79.1
ABBB (6)	0.1100	32	32.2
BBAA (7)	0.0408	14	12.0
BBAB (8)	0.0918	24	26.9
BBBB (9)	0.0374	11	11.0

GYS1 *gene effects*

The results of the GLM analysis of association between the *GYS1* gene and meat quality performance in pigs are summarised in Table 4. Differences among *Fok*I genotypes were only significant for pH of *m. semipinalis capitis*. No differences were detected for other meat quality traits. The *AB* pigs had significantly higher pH of *m. semipinalis capitis* than *AA* pigs, but there was no significant difference as compared with *BB*. This locus seemed to be significantly dominant in action and the dominance effects were -0.012 ± 0.007 for pH of *m. semipinalis capitis*.

Linkage analysis showed that the *Fok*I marker was significantly linked with the selected markers on SSC6. Two-point linkage analysis revealed linkage to microsatellite markers *SW1302* (recombination fraction = 0.25; LOD = 7.02) and *SW1473* (recombination fraction = 0.20; LOD = 9.76) on the sex-average linkage. The most probable order produced by Build option is as follows (Kosambi cM; sex-average values): *SW1302–26.9 – GYS1–21.3 – SW1473–23.3 – S0121–27.8 – SW322*. However, the QTL analysis showed that there was no significant QTL for meat quality traits.

ACOX1 *gene effects*

The results of the GLM analysis of association between the *ACOX1* gene and traits in pigs were summarised in Table 6. Significant effects of *ACOX1* alleles were found on meat colour value of *m. longissimus dorsi* (MCV1), meat colour value of *m. biceps femoris* (MCV2), meat marbling score

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Table 4 *Statistical analysis of association between* GYS1 Fok*I-RFLP genotypes with meat quality traits*

Traits	*Fok*I-RFLP genotype (mean ± s.e.)			Genetic effects (mean ± s.e.)	
	Genotype *AA* ($n = 102$)	Genotype *AB* ($n = 156$)	Genotype *BB* ($n = 52$)	Additive	Dominance
PHLD	6.328 ± 0.018	6.341 ± 0.014	6.376 ± 0.025	0.023 ± 0.015	0.004 ± 0.011
PHBF	6.417 ± 0.013	6.415 ± 0.010	6.429 ± 0.018	0.005 ± 0.011	0.003 ± 0.008
PHSC	6.426[a] ± 0.012	6.457 ± 0.010[b]	6.447[a,b] ± 0.017	0.009 ± 0.010	−0.012[*] ± 0.007
WLR, %	6.904 ± 0.369	6.703 ± 0.296	6.500 ± 0.523	−0.195 ± 0.323	0.006 ± 0.218
WHC, %	90.647 ± 0.509	90.825 ± 0.409	91.189 ± 0.721	0.261 ± 0.447	0.037 ± 0.300
MCV1	20.632 ± 0.289	20.271 ± 0.232	19.897 ± 0.409	−0.351 ± 0.254	0.010 ± 0.171
MCV2	19.263 ± 0.112	19.038 ± 0.090	19.181 ± 0.158	−0.034 ± 0.098	0.096 ± 0.066
MMS1	3.424 ± 0.018	3.413 ± 0.015	3.449 ± 0.026	0.010 ± 0.016	0.010 ± 0.011
MMS2	4.100 ± 0.017	4.096 ± 0.014	4.128 ± 0.024	0.010 ± 0.015	0.010 ± 0.010
IMF, %	3.105 ± 0.054	3.156 ± 0.043	3.175 ± 0.076	0.021 ± 0.047	−0.019 ± 0.032
WM, %	73.824 ± 0.075	73.771 ± 0.060	73.826 ± 0.106	0.005 ± 0.065	0.031 ± 0.044

[a,b]Within a row, means marked with different superscript letters are significantly different ($P < 0.05$).

of *m. longissimus dorsi* (MMS1), and meat marbling score of *m. biceps femoris* (MMS2). This locus seemed to be significantly over-dominant in action for meat colour value and meat marbling score, and pigs with genotype *AB* had significantly lower meat colour value, but higher meat marbling score as compared those with genotype *BB*. However, other important meat quality traits, such as water moisture and pH, were scarcely affected by *ACOX1* alleles.

Table 5 *Estimated effects (mean ± s.e.) of QTL for pH on pig chromosome 6*

Trait	Marker interval (cM)	F-value	Additive effect	Dominant effect
pHLD	*GYS1–SW1473* (33 cM)	2.29	0.024 ± 0.031	0.015 ± 0.023
pHBF	*SW1302–GYS1* (22 cM)	2.76	0.009 ± 0.017	0.012 ± 0.016
pHSC	*GYS1–SW1473* (29 cM)	3.92	0.011 ± 0.025	−0.018 ± 0.013

Discussion

The present work was based on the analysis of a F_2 segregation population derived from the intercross of Chinese Meishan and Large White pigs. Due to the significant phenotypic difference between Large White and Meishan pigs, the F_2 segregation population showed great variation in meat quality traits. The frequency of genotypes *AA*, *AB* and *BB* for *ACOX1* gene conformed to 1 : 2 : 1 Mendelian segregation, because the *A* allele was fixed in the founder Large White pigs and *B* allele fixed in the founder Chinese Meishan pigs.

Ultimate pH of pork is the most commonly used trait to assess pork quality. A higher level of acidity within the muscle (lower pH) causes muscle protein to denature and lose the ability to hold water. Therefore, meat with higher pH will tend to have more desirable characteristics (Malek *et al*., 2001). The pH of pork is correlated with glycogen content and glycolysis in postmortem muscle. GYS1 is a key enzyme of non-oxidative pathway of glucose metabolism and has been shown to strongly influence muscle glycogen content and glycolysis in skeletal muscle. This study demonstrates a significant genotype effect of *GYS1* on pHSC, which is in good agreement with physiological functions of *GYS1* gene. In order to discriminate between causal and neutral mutations in the F_2 design, we made use of the information provided by the neutral genetic markers located in the adjacent region of this mutation and conducted the QTL analysis. The peak of QTL for pH was located around the mutation of *GYS1* gene and F-ratio of QTL for pHSC was higher than that of pHLD and pHBF (Table 5). Therefore, the size of the *GYS1* genotype effects on the different muscle pH in the association study may mainly depend on the linked QTL effects. However, no significant QTL for pHSC was detected on this region although significant association of this mutation with pHSC was found in the single marker association. It may be due to several reasons. (1) The F_2 design has a great power to detect QTL provided by linkage disequilibrium, and also makes it difficult to discriminate between causal and neutral mutations. Therefore, a high percentage of false positives can be expected (Varona *et al*., 2005). (2) The number of animals we detected is not enough to demonstrate the true event. (3) The significant effects of *GYS1* gene were balanced by another QTL or marker with negative effects, as the QTL region was broad. All these need further verification.

ACOX1 is the first and rate-limiting enzyme in the peroxisomal fatty acid beta-oxidation pathway, suggesting this gene may be a potential candidate gene for the traits related to fat metabolism. IMF content is a major determinant of meat quality. After the elimination of the halothane mutation, the next limiting factor for meat quality would be IMF (Webb, 1998). The IMF can be measured by subjective and objective methods. Meat marbling score is one of subjective methods. The more the IMF content, the higher the meat marbling score. This study showed a significant effect of *ACOX1* on meat marbling score. Although

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Table 6 *Statistical analysis of association between* ACOX1 Pst*I-RFLP genotypes with meat quality traits*

Trait	*ACOX1-Pst*I-RFLP genotype (mean ± s.e.)			Genetic effects (mean ± s.e.)	
	Genotype *AA* ($n = 72$)	Genotype *AB* ($n = 159$)	Genotype *BB* ($n = 67$)	Additive	Dominance
PHLD	6.325 ± 0.022	6.354 ± 0.014	6.342 ± 0.022	0.008 ± 0.015	−0.010 ± 0.011
PHBF	6.400 ± 0.015	6.426 ± 0.01	6.423 ± 0.016	0.012 ± 0.011	−0.008 ± 0.008
PHSC	6.432 ± 0.015	6.444 ± 0.01	6.462 ± 0.015	0.016 ± 0.010	0.000 ± 0.007
WLR, %	6.634 ± 0.446	6.502 ± 0.298	7.482 ± 0.459	0.424 ± 0.319	0.278 ± 0.219
WHC, %	90.839 ± 0.616	91.19 ± 0.412	89.828 ± 0.633	−0.507 ± 0.441	−0.428 ± 0.303
MCV1	$20.304^{a,b}$ ± 0.349	20.115^{a} ± 0.233	21.08^{b} ± 0.359	0.396 ± 0.250	0.282^{*} ± 0.171
MCV2	$19.163^{a,b}$ ± 0.134	19.042^{a} ± 0.089	19.465^{b} ± 0.137	0.156 ± 0.096	0.132^{*} ± 0.066
MMS1	$3.413^{a,b}$ ± 0.022	3.449^{a} ± 0.015	3.373^{b} ± 0.023	−0.022 ± 0.016	-0.027^{*} ± 0.011
MMS2	$4.102^{a,b}$ ± 0.021	4.129^{a} ± 0.014	4.073^{b} ± 0.022	−0.015 ± 0.015	−0.020 ± 0.010
IMF, %	3.069 ± 0.066	3.176 ± 0.044	3.054 ± 0.068	−0.010 ± 0.047	−0.055 ± 0.032
WM, %	73.854 ± 0.091	73.784 ± 0.061	73.755 ± 0.094	−0.050 ± 0.045	−0.010 ± 0.042

^{a,b}Within a row, means marked with different superscript letters are significantly different ($P < 0.05$).

this SNP did not significantly contribute to the variation in IMF content in this population, the effect of this locus still approached the $P < 0.05$ statistical level (data not shown). As we have not performed the QTL analysis in this chromosome, we cannot determine whether the significant associations are due to the marker or due to other co-inherited blocks. However, the dominance effects were consistent within two F_2 populations except for the effects on MMS2 (data not shown).

The potential gain of MAS would be in terms of reduced costs for sib testing after slaughter and reduction in sophisticated meat quality measurements as well as additional improvement of meat quality by early information from genetic markers (Ovilo *et al*., 2006). At present, most of the market pigs are the hybrids among different specialised pig sire and dam lines. As for the dominant markers in the MAS programme, one of the alleles can be selected in sire line, and the other allele selected in the dam line, so the dominant effects can be realised in the hybrids. From this point of view, we can select the allele from Meishan pigs in the synthetic dam lines, and then the dam line can be crossbred with Large White pig sire lines. However, before the selection of these markers in the specialised dam lines, we should confirm the effects of those markers by comparing the meat quality traits of crossbred pigs carrying different genotypes, as the alternative allele was not completely fixed in the synthetic dam lines prior to the selection. In addition, we have to re-test for the effects in different crossbred populations, because it is likely that this polymorphism indirectly affects meat quality traits by being in linkage disequilibrium with another polymorphism that directly influences the quantitative traits analysed.

Conclusions

The results showed that the *Fok*I variants of the *GYS1* gene was significantly associated with pHSC in the association studies, but when taken into account the information provided by the neutral genetic markers on this chromosome, the peak of the QTL curve was located around this marker for pH, but it did not reach significance level. The effects of the *Pst*I variants in the ninth intron of the *ACOX1* gene polymorphism on marbling and IMF were consistent. These two loci seemed to be significantly dominant in action. Those dominant markers could be used in the MAS of crossbreeding of specialised pig sire and dam lines.

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