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A post-genome-wide association study validating the association of the glycophorin C gene with serum hemoglobin level in pig

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Objective: This study aimed to validate the statistical evidence from the genome-wide association study (GWAS) as true-positive and to better understand the effects of the glycophorin C (*GYPC*) gene on serum hemoglobin traits.
Methods: Our initial GWAS revealed the presence of two single nucleotide polymorphisms (SNPs) (ASGA0069038 and ALGA0084612) for the hemoglobin concentration trait (HGB) in the 2.48 Mb region of SSC15. From this target region, GYPC was selected as a promising gene that associated with serum HGB traits in pigs. SNPs within the *GYPC* gene were detected by sequencing. Thereafter, we performed association analysis of the variant with the serum hemoglobin level in three pig populations.
Results: We identified one SNP (g.29625094 T>C) in exon 3 of the *GYPC* gene. Statistical analysis showed a significant association of the SNP with the serum hemoglobin level on day 20 ($p<0.05$). By quantitative real-time polymerase chain reaction, the *GYPC* gene was expressed in eight different tissues.
Conclusion: These results might improve our understanding of GYPC function and provide evidence for its association with serum hemoglobin traits in the pig. These results also indicate that the *GYPC* gene might serve as a useful marker in pig breeding programs.

Keywords: Pig; Glycophorin C; Association; Hemoglobin Level; Expression

INTRODUCTION

Glycophorin C (GYPC) is an integral membrane glycoprotein. In humans, the GYPC gene encodes two erythrocyte surface sialoglycoproteins, glycophorin C (GPC) and glycophorin D (GPD) by initiating translation at two initiation codons on a single transcript [1,2]. GYPC participates in the mechanical steadiness of red blood cells. It also functions as the receptor for the *Plasmodium falciparum* (*P. falciparum*) erythrocyte-binding antigen, and receptor–ligand binding is responsible for the invasion of *P. falciparum* into human erythrocytes [3]. A deletion of exon 3 of the *GYPC* gene, which has been identified in Melanesians, changes the serologic phenotype of the Gerbich (Ge) blood group system, resulting in Ge negativity [4,5].

Our previous genome-wide association study (GWAS) showed the presence of two significant single nucleotide polymorphisms (SNPs), ASGA0069038 ($p = 9.59E-07$, $n = 681$) and ALGA0084612 ($p = 5.82 E-07$, $n = 681$) harbored within a 2.48 Mb stretch containing the *GYPC* gene of SSC15. From a statistically significant standpoint, there was an association with serum hemoglobin traits [6], supporting results of an earlier study that showed GYPC to regulate the mechanical stability of red blood cells [7]. According to comparative genomic studies, as well as significant signals in the GWAS, we postulate that the *GYPC* gene might be a good target gene for serum hemoglobin traits in pigs. The association of GYPC polymorphisms with serum hemoglobin traits in pigs has not been reported.

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To understand the relationship between the porcine *GYPC* gene and the serum hemoglobin level, we searched for genetic variants associating with serum hemoglobin traits. To achieve this, we performed an association study of novel SNPs within the *GYPC* gene. We identified two polymorphisms of the *GYPC* gene in pig, studied mRNA expression patterns, and considered their functions in red blood cells. We also performed association analysis between the SNPs and the serum hemoglobin level to estimate its potential effects in three pig breeds. Our results showed that the identified variant in the *GYPC* gene might be an important genetic factor implicated in the serum hemoglobin level in pig and that it might serve as a useful marker in breeding programs.

MATERIALS AND METHODS

Animal and tissue samples

This study employed three pig breeds including Landrace, Large White, and Songliao Black (a Chinese indigenous breed). They were sampled from the experimental farm of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China. Twenty-one-day-old pigs were vaccinated with classical swine fever live vaccine. Prior to vaccination, blood was collected from 20-day-old pigs, as well as from 35-day-old pigs. Ear tissue samples were also collected from all pigs for DNA extraction. Eight different tissues, including heart, liver, spleen, lung, kidney, muscle, stomach, and blood, were collected from three 35-day-old Landrace pigs. For each tissue, three fragments were collected, immediately frozen in liquid nitrogen and stored at –80°C for expression analysis.

Measurement of the serum hemoglobin level

All pigs were apparently healthy. Blood was collected according to the protocol approved by the Animal Welfare Committee of China Agricultural University (Permit number: DK997). The hemoglobin level was tested at the Beijing Xiyuan Hospital immediately after sample collection using a TEK-II mini automatic hemocyte analysor (Jiangxi Tekang Science and Technology, Nanchang, China) with a swine-specific parameter configuration.

Genomic DNA isolation and total RNA extraction

Genomic DNA was extracted from ear tissues of pigs by phenol/chloroform and ethanol precipitation [8]. The quality and quantity of all DNA samples were assessed by 1% agarose gel electrophoresis and NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA), respectively. Total RNA from the blood was isolated by a TRI Pure LS Reagent (Beijing BioTeke Corporation, Beijing, China). The total RNA from other tissues was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and quantified as indicated for DNA using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The integrity of the RNA samples were detected by 1% agarose gel electrophoresis before the first-strand cDNA was synthesized. RNA was purified and reversely transcribed into cDNA using the PrimerScript RT Reagent Kit with gDNA Eraser (TaKaRa Biotechnology Co., Ltd, Dalian, China) following the manufacturer's instructions.

SNP identification and genotyping of the porcine *GYPC* gene

Four pairs of polymerase chain reaction (PCR) primers were designed based on the porcine GYPC genomic sequence referring to the Sscrofa 10.2 primary assembly (ENSEMBLE Gene ID: ENSSSCG00000030984) to amplify all exons and partial adjacent introns (Table 1). DNA from 30 piglets was selected randomly to construct a DNA pool with equal DNA concentration of 50 ng/μL for each individual. PCRs were performed in a 25 μL volume containing 10 pmol of forward and reverse primers, 2.5 μL of 10× PCR buffer, 5 mM dNTPs, 50 ng of pooled DNA, 0.625 U Taq DNA polymerase (Takara Biotechnology Co. Ltd., China) and ddH_2O. The reaction conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 59°C to 60°C for 40 s, extension at 72°C for 40 s, and a final extension at 72°C for 10 min. All PCR fragments were purified with the Gel Extraction Mini Kit (Beijing Tiangen Biotechnology, China) and then three PCR products for each primer were sequenced using the ABI 3730xl DNA Analyzer (Applied Biosystems, CA, USA), and the identified SNPs were conducted by Chromas software (version 2.3.1) and DNAMAN (version 6.0). Genotyping of the identified SNPs was performed on 305 pigs by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) detection technology (Squenom MassARRAY; Bioyong Technologies Inc., Beijing, China).

Association analysis

Association analysis between the genotypes of the SNPs and the hemoglobin level was carried out by SAS software (version 9.1.3) based on the following mixed model:

$$y = X\beta+Zb+e$$

Where y was the vector of phenotype for the analyzed blood

Table 1. Primers and annealing temperatures used for PCRs of the porcine *GYPC* gene

Primers	Sequences 5′→3′	Anneal temperature (°C)	Product size (bp)
F1	TGACAATCCTGAGGGTCCA	60.0	450
R1	GGATCCCTGTGGATCTAGGG		
F2	CAGCTGTCAGCAGGTCTGAG	60.0	750
R2	CCCTGGGGTGATATTTGATG		
F3	CAGCTGTCAGCAGGTCTGAG	59.8	600
R3	GCCCATACCTCCTCCCTCTA		
F4	CTGGTCTGCCTTCTTCTGCT	59.5	640
R4	CATCCCTGGGAGGTGAAAC		

PCRs, polymerase chain reactions; GYPC, glycophorin C.

hemoglobin level, β was the vector of fixed effects parameter that included the breed and genotype effect, X was its incidence matrix, b was the mixed vector of random component that included the sires effect and dam effect within sires, and Z was its incidence matrix, and e was the vector of residual effect.

Tissues expression analysis of the porcine *GYPC* gene

Expression levels of mRNA in blood and eight different tissues of three 35-old-day Large White pigs were detected by real-time quantitative PCR using LightCycler 480 II instrument (Roche Diagnostics GmbH, Mannheim, Germany). The reaction system contained 10 pM each of the forward (F) and reverse (R) primers, 10 μL of 2× SYBR green I mixture, and 20 ng of cDNA in a final volume of 20 μL. Primer sequences for amplifying the *GYPC* gene were: F: 5'-GGGGAATAAGGAAGCGAGTC-3', R: 5'-CTGAAA CAACGGCAAACAAA-3′. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was served as an internal reference gene for normalization and the primers were: F: 5′- GTCC ACTGGTGTCTTCACGA-3′, R: 5′-GCTGACGATCTTGAGG GAGT-3′. The reaction conditions were as follows: 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. All testing values were carried out in triplicate and normalized to GAPDH by the $2^{-\Delta\Delta Ct}$ method [9].

RESULTS

Tissues expression analysis of the porcine *GYPC* gene

We applied the real-time quantitative PCR to identify the relative mRNA expression levels of porcine *GYPC* gene in eight different tissues. The results showed that mRNA of GYPC was expressed in all analyzed tissues. The highest level of expression was in blood, following are lung, spleen, liver, heart, stomach, kidney, and much lower expression level was found in skeletal muscle (Figure 1). The skeletal muscle is not animal's erythroid tissue, so the lower expression level was found. Our results were consistent with the research by Le et al [10], in which GYPC expressed both in erythroid and non erythroid tissues, but the level of transcription in erythroid is much higher than in non erythroid tissues in human.

Detection of polymorphisms and association analysis of the porcine *GYPC* gene

Sequence comparison of an identical PCR fragment from three pig breeds were detected one significant SNP (g.29625094 T>C) in exon 3 of the *GYPC* gene. The SNP was a synonymous mutation, meaning that there was no change in the sequence of the amino acids. The SNP (g.29625094 T>C) was genotyped in 305 piglets by applying the MALDI-TOF MS assay. The Hardy–Weinberg equilibrium for genotypic distributions, which employed the Chi-square (x^2) test, revealed that the alleles satisfied the requirements of the Hardy–Weinberg equilibrium in our experimental population (p>0.05). Genetic variation analysis also demonstrated that the frequencies were not significantly different among the three pig breeds (p>0.05) (Table 2). The allele T was dominant in Landrace and Large White; likewise, the TT genotype had higher frequencies. However, the allele C and the CC genotype had higher frequencies in Songliao Black.

Based on the aforementioned findings, we performed association analysis to confirm whether the identified SNP in the three pig breeds associates with the serum hemoglobin level.

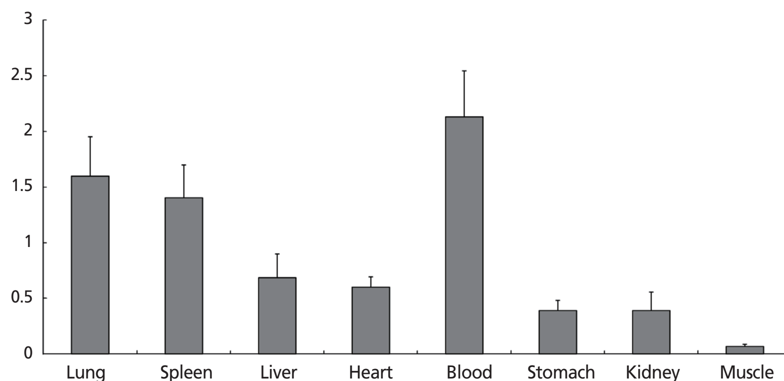

Figure 1. Relative quantification of expression levels of porcine GYPC mRNA in eight different tissues. Bars represent the mean±standard error (n = 3). The values were normalized to internal GAPDH expression. GYPC, glycophorin C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Genotype frequencies and allelic frequencies of *GYPC* gene determined by MALDI-TOF-MS in three pig populations

Breed	Number	Genotype frequencies			Allele frequencies	
		TT	TC	CC	T	C
Landrace	68	0.41	0.51	0.08	0.67	0.33
Large White	163	0.50	0.43	0.07	0.71	0.29
Songliao Black	74	0.23	0.31	0.46	0.39	0.61

GYPC, glycophorin C; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry.

We found a significant difference in the hemoglobin level among the three pig breeds (p<0.05). The serum hemoglobin level (days 20 and 35) in Large White was considerably lower than that in Landrace and Songliao Black, and Songliao Black had the highest hemoglobin level (days 20 and 35) among the three pig breeds (Table 3). These results indicate that genetic background can influence immune traits and that Chinese indigenous breeds might be immunologically superior to the other two western commercial pig breeds used in our study.

On the other hand, association analysis showed that the SNP (g.29625094 T>C) associated significantly with the serum hemoglobin level (days 20 and 35) (p = 0.0142; p = 0.3623) (Table 4). To determine the significance of the different genotypes acting on the serum hemoglobin level, multiple comparison tests were performed. The serum hemoglobin level (day 35) of pigs from the three genotypes was not significantly different. However, the serum hemoglobin level (day 20) of pigs from the genotype CC was significantly higher than that in pigs with the genotype TT (p<0.05) (Table 4). These results indicate that there is a likely association between the *GYPC* gene and the serum hemoglobin level, which should be confirmed in a larger population.

DISCUSSION

Hematological traits are important indicators of immune function in animals. They have been routinely examined as biomarkers of disease and various hematological related disorders in humans and other mammals [11]. Erythrocytes function mainly in the transport of oxygen and carbon dioxide [12], and their dysfunction associates with different disorders, including cancer and cardiovascular, metabolic, infectious and immune diseases [13]. In humans, the hemoglobin level is an independent predictor of adverse outcomes in women presenting with suspected ischemia. A decline in the hemoglobin level with a subsequent impaired oxygen-carrying ability in the setting of limited coronary flow by either mechanism can worsen ischemia and related symptoms [14].

Although important genetic effects of erythroid traits exist, the genetic architecture of erythroid traits is still poorly understood. Thus, identifying causal genes underlying these traits provide novel targets for risk assessment. With the development of high throughput SNP genotyping technologies, the GWAS has become a routine approach for investigating mutations underlying complex traits. Results from the GWAS have been successfully applied in identifying functional genes involved in important economical traits in pigs [15,16]. Numerous candidate SNP loci associating with respective target traits have been identified by the GWAS. However, the GWAS is only the first step in the identification of relevant genes. These findings require additional studies to ascertain the causal variants by functional verification [17,18]. Up until now, most genome-wide association studies have identified genomic regions or SNPs associating with erythroid traits in pigs [11,19]. In our initial GWAS, the *GYPC* gene was identified [6].

GPC is a glycoprotein found in the membrane of human red blood cells (RBC). It plays a key role in the regulation of RBC shape and membrane mechanical properties by providing a membrane linkage site for cytoskeletal proteins [1]. The absence of GPC in RBC membranes associates with hereditary ellyptocytosis. In humans, *P. lasmodium falciparum* invades erythrocytes via glycophorin C [20]. GPC is encoded by the *GYPC* gene. The human *GYPC* gene is located on the long arm of chromosome 2q14-q21, which is 13.5 kb long and comprised of four exons [21]. The porcine *GYPC* gene consists of three exons; it is located on chromosome 15. Because a significant signal was found for the *GYPC* gene in our initial GWAS, the *GYPC* gene might be a target gene for serum hemoglobin traits in pigs. Our association analysis results also showed that *GYPC* gene might be used as a genetic marker with significant effects on the blood hemoglobin level in pig disease resistance breeding. Furthermore, the detected SNP was not a missense mutation. Instead, it was a “silent” polymorphism change based on substrate specificity

Table 3. Association analysis of hemoglobin level in three pig breeds

Traits	Breeds (Means±standard error of means)		
	Landrace (n = 68)	Large White (n = 163)	Songliao Black (n = 74)
Hemoglobin level (day 20)	115.94±27.73[a]	107.41±16.11[b]	118.94±27.29[a]
Hemoglobin level (day 35)	117.35±19.42[a]	110.10±26.29[b]	119.46±28.31[a]

[a,b] Statistically different of least square means (p<0.05).

Table 4. Association analysis and multiple tests of the SNP (g.29625094 T>C) of *GYPC* gene with hemoglobin level in three pig populations

Traits	Genotypes (Means±standard error of means)			p value
	TT (n = 126)	TC (n = 128)	CC (n = 51)	
Hemoglobin level (day 20)	110.53±2.29[a]	112.80±2.76[a]	116.62±2.01[b]	0.0142*
Hemoglobin level (day 35)	116.59±2.57	117.58±02.96	118.97±3.18	0.3623

SNP, single nucleotide polymorphism; GYPC, glycophorin C.
[a,b] Statistically different of least square means (* p<0.05).

[22]. Similar SNPs have also been reported to influence protein expression by alternating or increasing in mRNA stability [23]. Thus, further exploration of the genetic relationship of expression patterns with different GYPC genotypes among different pig groups will be more meaningful. In addition, the number of pigs in our study was limited, additional studies are needed to confirm the relationship between the SNP and the serum hemoglobin level in a large pig population before the gene is used for selection.

In summary, the *GYPC* gene was expressed widely in eight tissues. One SNP in exon 3 of the *GYPC* gene was identified, and association analysis showed that the SNP (g.29625094 T>C) was significantly associated with the serum hemoglobin level in three pig breeds. Our results indicate that the *GYPC* gene, with effects on the serum hemoglobin level in pig, might be a useful marker in breeding programs. Our results may also help to understand its biological function better.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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