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Research article

Association of *FTH* and *EPOR* gene polymorphisms with litter size traits in pigs

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Abstract

Ferritin heavy chain (*FTH*) and erythropoietin receptor (*EPOR*) are responsible for the iron homeostasis and the erythropoiesis that correlate to the reproductive systems. This study aimed to examine an association of the porcine *FTH* and *EPOR* genes with litter size traits in Large White and Landrace pigs. The porcine *FTH* g.9537834G > A was significantly associated with the total number born (TNB) trait in these pig populations ($P < 0.05$). The porcine *FTH* g.9537855T > C was significantly associated with the TNB trait in Large White sows ($P < 0.05$) as well as the TNB and the number of birth alive (NBA) traits in Landrace sows ($P < 0.05$). The porcine *EPOR* g.70066473C > T was significantly associated with the TNB trait in Large White sows ($P < 0.05$) as well as the TNB, NBA, and the number of piglets weaned alive (NWA) traits in Landrace sows ($P < 0.05$). Moreover, the accumulated favorable alleles of these three SNPs were increasingly associated with TNB trait in Large White sows ($P < 0.05$) and TNB, NBA, and NWA traits in Landrace sows ($P < 0.05$). These findings suggest that porcine *FTH* and *EPOR* genes may contribute to the reproductive processes of pigs with regards to litter size and confirm the importance of these genes as candidate genes for improving litter size in pigs.

Keywords: *EPOR*, *FTH*, Litter size, Pig, Polymorphisms

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INTRODUCTION

Litter size traits are among the important reproduction traits in industrial pig production. Currently, genome-wide association study (GWAS) and transcriptomic approaches have been used to identify quantitative trait loci (QTL) and to assess various candidate genes for reproductive traits in pigs (Onteru et al., 2012; Trenhaile et al., 2016; Verardo et al., 2016; Wu et al., 2018). Numerous significant QTL regions for the total number of piglets born (TNB), number of piglets born alive (NBA), number of stillborn, and mummified fetus traits are located on SSC2 at positions 8.30 to 9.19 Mb and 82.82 to 104.68 Mb (Onteru et al., 2012; Trenhaile et al., 2016). Several potential candidate genes for litter size traits of these regions have been reported in pig populations e.g., arylsulfatase family member K (*ARSK*), purinergic receptor P2X 3 (*P2X3R*), G protein-coupled receptor 150 (*GPR150*), metallo-β-lactamase domain containing 2 (*MBLAC2*), transmembrane protein 161B (*TMEM161B*), and RAS p21 protein activator 1 (*RASA1*) (Onteru et al., 2012; Trenhaile et al., 2016). Interestingly, the ferritin heavy chain (*FTH*) and erythropoietin receptor (*EPOR*) genes are also located on SSC2 (at positions 9.53 and 70.06 Mb, respectively) closely to QTL regions for reproductive traits of pigs. Moreover, previous studies demonstrated that their gene functions are related to embryonic survival in mammalian species (Kieran et al., 1996; Ferreira et al., 2000; Vallet et al., 2005; Li et al., 2015). Thus, the *FTH* and *EPOR* genes are important to the reproductive system in mammals.

Ferritin is a major iron storage protein in organisms and plays an important role in the regulation of iron homeostasis and required for numerous cellular functions (Soheilykhah et al., 2017). Ferritin consists of the heavy and light chains, encoded by *FTH* (also known as *FTH*1) and *FTL* (also known as *FTL1*) genes, respectively (Huang et al., 2019). The FTH subunit has ferroxidase activity for converting Fe(II) to Fe(III), while the FTL subunit promotes iron nucleation and increases ferritin stability (Li et al., 2015). The transcript of the porcine *FTH* gene is 866 bp in length. It consists of five exons and four introns and encodes for a 181-amino acid peptide (ENSSSCT00000050390.2; https://asia.ensembl.org/index.html, accessed on 2 September 2021). The porcine *FTH* gene is located near the QTL regions for total number born (9.3 Mb) and the number of stillborn (10.2-10.5 Mb) (Onteru et al., 2012; He et al., 2017). Moreover, highly polymorphic sites of the porcine *FTH* gene have been characterized (https://asia.ensembl.org/index.html, accessed on 2 September 2021). The expression levels of *FTH* mRNA and the protein are regulated by progesterone and are increased in uterine stromal cells during pregnancy (Zhu et al., 1995). These pieces of evidence suggest that the FTH subunit plays an important role during embryogenesis (Li et al., 2015). Thus, the *FTH* gene may be critical for the survival of the embryo during gestation.

EPOR is a transmembrane cell surface protein or receptor for erythropoietin (EPO) and plays an essential role in erythropoiesis and other cellular processes (He et al., 2019). EPO is a hematopoietic cytokine produced in the fetal liver and adult kidney that stimulates erythropoiesis (Lombardero et al., 2011). The binding of EPO with EPOR leads to a signaling cascade triggering several genes responsible for the proliferation and differentiation of erythroid progenitors into mature red blood cells (Vočanec et al., 2019). The

failure of erythropoiesis may be involved in fetal mortality and reduction of litter size. Fetal erythropoiesis is impaired under crowded intrauterine conditions (Pearson et al., 1998). The accelerated erythropoiesis to the red blood cell maturity occurs in fetuses during the early pregnancy of the Chinese Meishan pig, that a greater number of fetal survival and a higher uterine capacity (Vallet et al., 2003). Therefore, faster blood cell development could be beneficial to fetal survival and enhance the uterine capacity of pigs. Therefore, the *EPOR* plays an important role in fetal survival by promoting the maturation of red blood cells in pigs (Zhang et al., 2011a). The transcript of *EPOR* gene is 1871 bp in length. It consists of eight exons and seven introns and encodes for a 509-amino acid peptide (ENSSSCT00000014873.4, https://asia.ensembl.org/index.html, accessed on 2 September 2021). The porcine *EPOR* gene is located near the QTL regions for the number of mummified pigs (73.9-75.0 Mb), litter birth weight (76.5 Mb), total of number born (84.3 Mb), and total number born alive (87.8 Mb) (Li et al., 2011; Onteru et al., 2012; He et al., 2017; Wu et al., 2018). Moreover, highly polymorphic sites of the porcine *EPOR* gene have been reported in the Ensembl database (https://asia.ensembl.org/index.html, accessed on 2 September 2021). Numerous SNPs of the porcine *EPOR* gene have been used to test for an association with litter size traits in Western crossbred and Chinese indigenous pig breeds (Vallet et al., 2005; Zhang et al., 2011a; 2011b).

These shreds of evidence suggest that the porcine *FTH* and *EPOR* genes are responsible for iron homeostasis and erythropoiesis. Moreover, their functions are critical for embryogenesis and fetal survival during early pregnancy, as well as their positions, are closely located with the QTLs for reproductive traits of pigs. Therefore, the porcine *FTH* and *EPOR* genes can be regarded as candidate genes for litter size traits in pigs. However, currently there is no evidence of the relationship between the porcine *FTH* and *EPOR* genes with litter size traits in pigs. The objective of this study was to examine whether the polymorphisms of the porcine *FTH* and *EPOR* genes as well as their accumulated favorable alleles are associated with litter traits in commercial pig breeds.

MATERIALS AND METHODS

Animals and DNA extraction

Blood samples were collected from totally of 312 Large White and 339 Landrace sows. These sows were obtained from the Betagro Group (Thailand). The sows were fed a corn-soybean-based diet containing 16% crude protein and 3388 kcal/kg digestible energy and were maintained in closed houses with evaporative cooling systems. Litter size data were available from 974 Large White and 1068 Landrace litters (1st to 8th parities) and were recorded in terms of total number born (TNB), number born alive (NBA), the number of piglets weaned alive (NWA), mean birth weight of the piglets (MBW), and mean weight of piglets at weaning (21 days, MWW) (Norseeda et al., 2021a; 2021b). Genomic DNA was extracted from blood samples using the Chelex method (Walsh et al., 2013) and store at 4°C for further analysis. The experimental procedures were approved by the Animal Ethics Committee of Chiang Mai University, Thailand (2562/AG-0001).

Genotyping

Three polymorphic sites of the porcine *FTH* g.9537834G > A (rs55619215), *FTH* g.9537855T > C (rs55619227), and *EPOR* g.70066473C > T (rs81208915) loci were used to genotype in two commercial pig populations. The porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C loci were corresponding to 3′-untranslated region (3′-UTR) SNP (*Msp*I-c.677T > C and MspI-c.699T > C) of the porcine *FTH* gene as ascribed by a previous study (Pripwai and Mekchay, 2012). Moreover, the porcine *EPOR* g.70066473C > T locus was corresponding to a SNP of intron 4 (g.2373C > T) of the porcine *EPOR* gene (Vallet et al., 2005; Zhang et al., 2011a). These three SNPs were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The specific primers of the porcine *FTH* and *EPOR* genes were designed using nucleotide sequence information (GenBank accession number: NC_010444.4), as shown in Table 1. A mismatched primer was designed to create a recognition site of the restriction enzyme for genotyping (Table 1). The PCR products were amplified in a total volume of 20 µL containing 50 ng of a genomic DNA sample, 1×$(NH_4)_2$ SO_4 buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer and 0.2 U *Taq* DNA polymerase (Thermo Scientific, Hanover, MD, USA). The amplification conditions were as follows: 94°C for 3 min for initial denaturing and 35 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 5 min. The PCR fragments were digested with the restriction enzymes (Thermo Scientific, Hanover, MD, USA) as shown in Table 1. The digested PCR fragments were separated on 6% polyacrylamide gel electrophoresis and the gels were visualized by ethidium bromide staining.

Table 1 Primer sequences and restriction enzymes used for genotyping of porcine *FTH* and *EPOR* genes.

SNPs	Location	Primer sequences (5′to 3′)	PCR size (bp)	Ta (°C)	Restriction enzyme
FTH g.9537834G > A, *FTH* g.9537855T > C	3′-UTR	F: CATTGAGACGCATTACCTG R: GTACACTAAGGAAAGAACTG	281	58	*Msp*I
EPOR g.70066473C > T	Intron 4	F*: GCCTCCTGCTTTCATTGCGTA R: GGCCAAGAAGGGCATGTTG	104	58	*Rsa*I

*Mismatched base is underlined to generate a recognition site of the restriction enzyme for genotyping. Ta: annealing temperature; 3′-UTR: 3′-untranslated region; SNPs: single nucleotide polymorphisms; PCR: polymerase chain reaction.

Statistical analysis

Allele and genotype frequencies were calculated for each SNP locus. A chi-square test was conducted to examine the populations for Hardy-Weinberg equilibrium (HWE). Association analysis of the *FTH* and *EPOR* polymorphisms was examined using the mixed model as follows: $Y_{ijklm} = \mu + P_i + YS_j + G_k + A_l + e_{ijklm}$, where Y_{ijklm} is representative of the observations of the phenotype values, µ represents the average normalized record of population, P_i represents the fixed effect of the parities (i = 1 and ≥ 2), YS_j represents the fixed effect of the year-seasons (j = 1-8), G_k is representative of the fixed effect of the porcine *FTH* or *EPOR* genotypes (k = 1-3), or the accumulated favorable alleles for the *FTH* g.9537834G > A, *FTH* g.9537855T > C, and *EPOR* g.70066473C > T (k = 0-6), A_l is representative of the random effect of the animal, and e_{ijklm} represents the residual error. Besides, the additive effect was analyzed as half difference

between the two homozygous genotypes and the dominance effect was calculated as the deviation of the heterozygous genotype effect from the mean effect of the two homozygous genotypes (Muñoz et al., 2007; Norseeda et al., 2021c). The least square mean values between genotype groups for each locus were compared using the least significant differences (LSD) test ($P < 0.05$).

In silico analysis

The *in silico* analysis was used to predict the effects of SNPs in the 3′-UTR of porcine *FTH* gene on miRNA-binding site. The 3′-UTR of the porcine *FTH* mRNA (GenBank accession no. NM_213975) was aligned with the 3′-UTR of the human *FTH* mRNA (NM_002032) using the CLUSTALW software (www.genome.jp/tools-bin/clustalw) and the putative microRNA targets on the 3′-UTR of the human *FTH* gene were predicted using the TargetScanHuman v8.0 database (www.targetscan.org).

RESULTS

Polymorphisms of porcine *FTH* and *EPOR* genes

Three polymorphic sites of the porcine *FTH* g.9537834G > A, *FTH* g.9537855T > C, and *EPOR* g.70066473C > T loci were found to be segregating in these two commercial pig populations. The porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C loci were amplified with the same primers and were detected with the restriction enzyme *Msp*I. The PCR-PFLP patterns of two porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C polymorphisms are shown in Figure 1. The porcine *EPOR* g.70066473C > T polymorphism was detected with the restriction enzyme *Rsa*I. Genotype patterns for the porcine *EPOR* g.70066473C > T polymorphism are indicated in Figure 2.

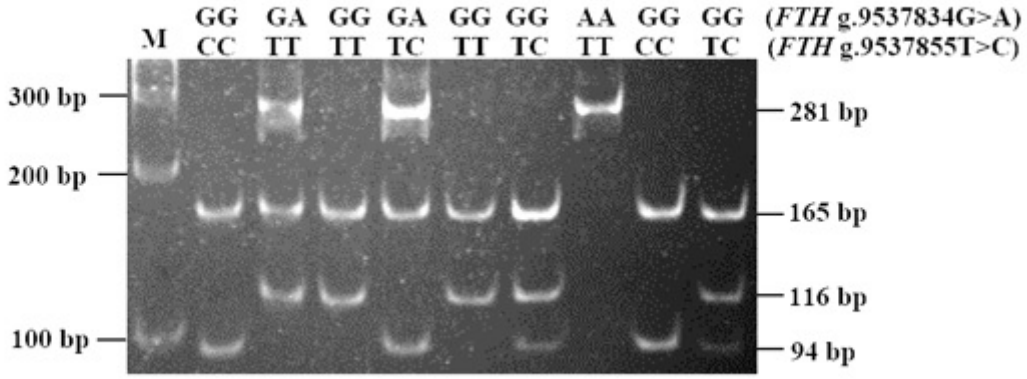

Figure 1 Genotyping SNPs of the porcine *FTH* gene at g.9537834G > A and g.9537855T > C loci with *Msp*I. The molecular marker of 100 bp DNA ladder (M) and the genotypes of *FTH* are indicated at the top of each lane. Fragment 281 bp is indicated for *FTH* g.9537834A and *FTH* g.9537855T alleles. Two fragments of the 165 and 116 bp fragments are indicated for *FTH* g.9537834G and *FTH* g.9537855T alleles, and three fragments of 165, 94, and 22 bp for *FTH* g.9537834G and *FTH* g.9537855C alleles. Notably, the 22 bp fragment is not shown in the gel.

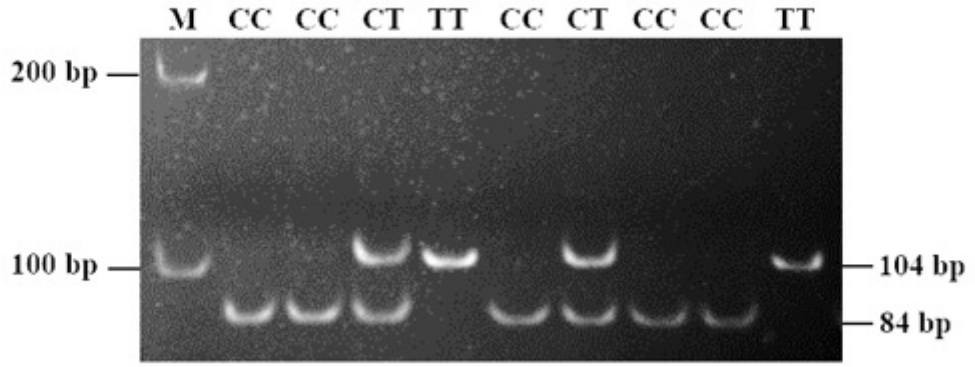

Figure 2 Genotyping SNP of the porcine *EPOR* gene at g.70066473C > T locus with *Rsa*I. The molecular marker of 100 bp DNA ladder (M) and the genotypes of *EPOR* are indicated at the top of each lane. Fragment 104 bp is indicated for allele T and two fragments of 84 and 20 bp for allele C. Notably, the 20-bp fragment is not shown in the gel.

Genotype and allele frequencies

The genotype and allele frequencies of the porcine *FTH* and *EPOR* genes are shown in Table 2. Two polymorphic sites of the porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C loci were found to be segregating in the Large White and Landrace sows. Six patterns of PCR-PFLP fragments of *FTH* g.9537834G > A and *FTH* g.9537855T > C loci were observed in these two pig populations. At the *FTH* g.9537834G > A and *FTH* g.9537855T > C loci, each of the three genotypes were exhibited. The *FTH* g.9537834G and *FTH* g.9537855T alleles were more frequent in the Large White and Landrace sows. Moreover, the porcine *EPOR* g.70066473C > T polymorphism was found to be segregating in these two pig populations. At the *EPOR* g.70066473C > T locus, three genotypes were observed. The *EPOR* g.70066473C allele was more frequent in these sows. The chi-square test showed that the genotype distributions of the porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C loci within Large White and Landrace sows deviated from the Hardy-Weinberg equilibrium specifications. Whereas the genotype distributions of the porcine *EPOR* g.70066473C > T locus within Large White and Landrace sows were in agreement with the Hardy-Weinberg equilibrium specifications.

Table 2 Genotype and allele frequencies of porcine *FTH* and *EPOR* genes in Large White and Landrace sows.

SNPs	Breeds	n	Genotype frequencies			Allele frequencies1		P–value[2]
			AA	AB	BB	A	B	(χ^2)
FTH g.9537834G > A	Large White	312	0.85	0.11	0.04	0.90	0.10	0.001
	Landrace	339	0.83	0.09	0.08	0.87	0.13	0.001
FTH g.9537855T > C	Large White	312	0.80	0.06	0.14	0.83	0.17	0.001
	Landrace	339	0.74	0.08	0.18	0.78	0.22	0.001
EPOR g.70066473C > T	Large White	310	0.65	0.29	0.06	0.80	0.20	0.113
	Landrace	336	0.58	0.34	0.08	0.75	0.25	0.218

[1]Allele A represents wild type alleles of the *FTH* g.9537834G, *FTH* g.9537855T, and *EPOR* g.70066473C for each locus and allele B represents mutate alleles of the *FTH* g.9537834A, *FTH* g.9537855C, and *EPOR* g.70066473T.
[2]P–value is considered a significant level of the chi-square (χ^2) test for Hardy–Weinberg equilibrium of each locus.

Associations of porcine *FTH* and *EPOR* genes with litter size traits

The association of the porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C polymorphisms with litter size traits is shown in Tables 3 and 4. There was no significant association of the *FTH* g.9537834G > A with litter size traits in the first parity of Large White and Landrace sows. However, the *FTH* g.9537834G > A was significantly associated with the TNB trait in later parities of Large White and Landrace sows ($P < 0.05$). Notably, sows with the GG and GA genotypes had higher TNB values than sows with the AA genotype (+1.23 TNB). In addition, the significant additive effect for the TNB trait was observed in later parities of Large White and Landrace sows ($P < 0.05$). No significant association of porcine *FTH* g.9537855T > C with litter size traits was found in the first parity of Large White and Landrace sows. However, the *FTH* g.9537855T > C was significantly associated with the TNB trait in later parities of Large White sows ($P < 0.05$) as well as the TNB ($P < 0.01$) and NBA traits ($P < 0.05$) in later parities of Landrace sows. Notably, sows with the CC genotype had higher TNB and NBA values than sows with the TT and TC genotypes (+0.93 TNB and +0.81 NBA). Additionally, the significant additive effect for the TNB ($P < 0.01$) and NBA traits ($P < 0.05$) was found in later parities of Landrace sows.

Table 3 Association of porcine *FTH* g.9537834G > A locus with litter size traits.

Breeds	Parity	Traits[1]	Genotypes (Means±SE)[2]			Additive	Dominance
			GG	GA	AA		
Large White	First parity	n	265	33	14		
		TNB	11.39±0.30	12.53±0.81	12.50±1.19	-0.55±0.61	0.59±1.01
		NBA	9.67±0.29	10.85±0.78	11.41±1.14	-0.87±0.58	0.29±0.97
		NWA	8.75±0.28	9.48±0.74	8.77±1.09	-0.01±0.55	0.71±0.92
		MBW	1.39±0.02	1.27±0.05	1.35±0.07	0.01±0.04	-0.09±0.06
		MWW	6.53±0.03	6.65±0.09	6.64±0.14	-0.01±0.07	0.11±0.12
	Later parities	n	553	75	34		
		TNB	12.08±0.52^{b}	11.82±0.67^{b}	10.39±0.97^{a}	0.84±0.41*	0.58±0.63
		NBA	11.00±0.59	11.00±0.73	9.99±1.04	0.51±0.43	0.50±0.63
		NWA	10.37±0.59	10.82±0.72	9.76±1.05	0.31±0.44	0.74±0.64
		MBW	1.47±0.05	1.46±0.06	1.62±0.09	-0.07±0.04	-0.08±0.06
		MWW	6.45±0.05	6.48±0.06	6.59±0.09	-0.07±0.04	-0.04±0.05
Landrace	First parity	n	280	31	28		
		TNB	10.46±0.27	10.80±0.70	9.62±0.73	0.42±0.37	0.76±0.77
		NBA	9.05±0.30	9.15±0.79	8.49±0.82	0.28±0.41	0.38±0.86
		NWA	8.33±0.30	8.83±0.77	7.46±0.79	0.43±0.40	0.93±0.84
		MBW	1.53±0.02	1.52±0.07	1.53±0.06	0.01±0.03	-0.01±0.07
		MWW	6.46±0.03	6.46±0.08	6.47±0.08	-0.01±0.04	-0.01±0.09
	Later parities	n	593	74	62		
		TNB	11.16±0.37^{b}	10.85±0.49^{b}	10.10±0.54^{a}	0.53±0.22*	0.23±0.44
		NBA	9.47±0.44	9.49±0.55	9.15±0.61	0.15±0.23	0.18±0.47
		NWA	9.10±0.44	9.01±0.56	8.99±0.62	0.06±0.24	-0.03±0.48
		MBW	1.60±0.03	1.59±0.04	1.57±0.05	0.01±0.03	0.01±0.04
		MWW	6.55±0.05	6.57±0.06	6.65±0.07	-0.05±0.02	-0.03±0.04

[1]n: number of investigated litters, TNB: total number born, NBA: number born alive, NWA: number of piglets weaned alive, MBW: mean birth weight of the piglets, MWW: mean weight of piglets at weaning. MBW and MWW traits are expressed in kg.
[2]Means±SE represent least square means±standard error. Values in each row with differing superscripts are considered significantly different ($^{a, b}$ $P < 0.05$, * $P < 0.05$).

Table 4 Association of porcine *FTH* g.9537855T > C locus with litter size traits.

Breeds	Parity	Traits[1]	Genotypes (Means±SE)[2]			Additive	Dominance
			TT	TC	CC		
Large White	First parity	n	249	20	43		
		TNB	11.43±0.33	11.56±0.88	12.11±0.68	-0.33±0.36	-0.21±0.97
		NBA	9.72±0.31	10.57±0.85	10.05±0.66	-0.16±0.34	0.68±0.93
		NWA	8.69±0.29	9.83±0.79	8.80±0.62	-0.05±0.33	1.09±0.87
		MBW	1.37±0.02	1.41±0.06	1.34±0.04	0.01±0.02	0.05±0.06
		MWW	6.55±0.04	6.53±0.10	6.55±0.08	0.01±0.04	-0.01±0.11
	Later parities	n	522	48	92		
		TNB	10.77±0.56^{a}	11.35±0.80^{ab}	11.94±0.72^{b}	-0.59±0.27	-0.01±0.66
		NBA	9.91±0.60	9.54±0.82	10.12±0.75	-0.10±0.27	-0.04±0.65
		NWA	9.47±0.58	9.79±0.79	9.60±0.71	-0.06±0.24	0.25±0.60
		MBW	1.46±0.05	1.52±0.07	1.40±0.06	0.03±0.02	0.08±0.06
		MWW	6.46±0.05	6.40±0.06	6.46±0.06	0.01±0.02	-0.06±0.04
Landrace	First parity	n	251	28	60		
		TNB	10.30±0.28	10.19±0.66	11.01±0.47	-0.35±0.24	-0.47±0.68
		NBA	8.97±0.32	8.42±0.74	9.48±0.52	-0.25±0.26	-0.80±0.76
		NWA	8.27±0.03	8.33±0.72	8.42±0.51	-0.07±0.26	-0.01±0.74
		MBW	1.53±0.02	1.52±0.06	1.51±0.04	0.01±0.02	-0.01±0.06
		MWW	6.44±0.03	6.46±0.07	6.51±0.05	-0.03±0.03	-0.02±0.07
	Later parities	n	531	65	133		
		TNB	10.49±0.45^{A}	10.88±0.57^{AB}	11.67±0.55^{B}	-0.59±0.19**	-0.02±0.45
		NBA	9.09±0.49^{a}	9.33±0.59^{ab}	10.02±0.57^{b}	-0.46±0.19*	-0.22±0.46
		NWA	8.78±0.48	8.93±0.58	9.59±0.55	-0.41±0.19	-0.24±0.44
		MBW	1.62±0.03	1.59±0.05	1.58±0.04	0.02±0.02	-0.01±0.03
		MWW	6.56±0.05	6.58±0.06	6.54±0.06	0.01±0.02	0.03±0.04

[1]n: number of investigated litters, TNB: total number born, NBA: number born alive, NWA: number of piglets weaned alive, MBW: mean birth weight of the piglets, MWW: mean weight of piglets at weaning. MBW and MW W traits are expressed in kg. [2]Means±SE represent least square means±standard error. Values in each row with differing superscripts are considered significantly different ($^{a, b}$ $P < 0.05$, $^{A, B}$ $P < 0.01$, * $P < 0.05$, ** $P < 0.01$).

Association of the porcine *EPOR* g.70066473C > T polymorphism with litter size traits is shown in Table 5. There was no significant association of porcine *EPOR* g.70066473C > T with any litter size traits in the first parity of Large White and Landrace sows. However, the porcine *EPOR* g.70066473C > T was significantly associated with the TNB trait in later parities of Large White sows ($P < 0.05$). Notably, sows with the TT genotype had higher TNB values than sows with the CC and CT genotypes (+2.25 TNB). The significant additive effect for TNB trait was observed in later parities of Large White sows ($P < 0.01$). Furthermore, the porcine *EPOR* g.70066473C > T was significantly associated with the TNB ($P < 0.01$), NBA ($P < 0.01$), and NWA traits ($P < 0.05$) in later parities of Landrace sows. Notably, sows with the CT and TT genotypes had higher TNB, NBA, and NWA values than sows with the CC genotype (+1.31 TNB, +1.20 NBA, and +0.9 NWA). The significant additive effect for TNB ($P < 0.01$), NBA ($P < 0.01$), and NWA traits ($P < 0.05$) was detected in later parities of Landrace sows.

Table 5 Association of porcine *EPOR* g.70066473C > T locus with litter size traits.

Breeds	Parity	Traits[1]	Genotypes (Means±SE)[2]			Additive	Dominance
			CC	CT	TT		
Large White	First parity	n	202	89	19		
		TNB	10.94±0.38	11.74±0.67	12.35±1.17	-0.70±0.61	0.09±0.86
		NBA	9.17±0.38	10.36±0.66	10.26±1.16	-0.54±0.61	0.64±0.85
		NWA	8.30±0.35	9.03±0.62	8.60±1.08	-0.14±0.56	0.57±0.79
		MBW	1.39±0.02	1.39±0.04	1.39±0.07	0.01±0.03	-0.01±0.05
		MWW	6.58±0.04	6.61±0.08	6.34±0.14	0.11±0.07	0.15±0.10
	Later parities	n	421	192	44		
		TNB	11.49±0.46^{a}	11.60±0.60^{a}	13.80±0.99^{b}	-1.16±0.47**	-1.04±0.64
		NBA	10.36±0.45	10.42±0.58	11.45±0.95	-0.57±0.45	-0.50±0.61
		NWA	9.75±0.45	9.93±0.57	11.01±0.93	-0.63±0.42	-0.45±0.58
		MBW	1.47±0.03	1.43±0.04	1.46±0.07	0.01±0.03	-0.03±0.04
		MWW	6.62±0.03	6.60±0.05	6.65±0.08	-0.01±0.03	-0.03±0.04
Landrace	First parity	n	195	114	27		
		TNB	9.36±0.43	9.78±0.49	8.65±0.78	0.35±0.36	0.77±0.48
		NBA	8.03±0.48	8.83±0.55	7.60±0.87	0.21±0.40	1.01±0.53
		NWA	7.46±0.48	8.15±0.54	6.70±0.86	0.37±0.40	1.06±0.53
		MBW	1.61±0.03	1.57±0.04	1.61±0.06	0.01±0.03	-0.04±0.04
		MWW	6.39±0.04	6.40±0.05	6.35±0.08	0.02±0.04	0.02±0.05
	Later parities	n	405	249	65		
		TNB	10.51±0.37^{A}	11.20±0.41^{B}	12.47±0.63^{C}	-0.98±0.29**	-0.29±0.38
		NBA	9.15±0.39^{A}	9.95±0.42^{B}	10.76±0.64^{B}	-0.08±0.29**	0.01±0.38
		NWA	8.86±0.37^{a}	9.44±0.41^{b}	10.08±0.64^{b}	-0.06±0.29*	-0.03±0.38
		MBW	1.61±0.02	1.59±0.03	1.56±0.05	0.02±0.02	0.01±0.03
		MWW	6.62±0.04	6.61±0.04	6.58±0.07	0.03±0.03	0.01±0.03

[1]n: number of investigated litters, TNB: total number born, NBA: number born alive, NWA: number of piglets weaned alive, MBW: mean birth weight of the piglets, MWW: mean weight of piglets at weaning. MBW and MWW traits are expressed in kg.
[2]Means±SE represent least square means±standard error. Values in each row with differing superscripts are considered significantly different ($^{a, b}$ $P < 0.05$, $^{A, B, C}$ $P < 0.01$, * $P < 0.05$, ** $P < 0.01$).

Moreover, the effects of the accumulated favorable alleles of the porcine *FTH* g.9537834G > A, *FTH* g.9537855T > C, and *EPOR* g.70066473C > T polymorphisms on litter size traits are shown in Table 6. There was no significant association of the accumulated favorable alleles with any litter size traits in the first parity of the sows. Nevertheless, a significant association of these accumulated favorable alleles with the TNB trait was found in later parities of Large White sows ($P < 0.05$). Moreover, a significant association of the accumulated favorable alleles with the TNB, NBA, and NWA traits was found in later parities of Landrace sows ($P < 0.05$). Interestingly, an increased number of favorable alleles (*FTH* g.9537834G, *FTH* g.9537855C, and *EPOR* g.70066473T) was associated with litter size traits in these sows.

Table 6 Association of genotype combinations of porcine *FTH* g.9537834G > A, *FTH* g.9537855T > C, and *EPOR* g.70066473C > T with litter size traits.

Breeds	Parity	Traits[1]	Number of favorable alleles (Means±SE)[2]						
			0	1	2	3	4	5	6
Large White	First parity	n	14	16	153	57	44	15	11
		TNB	12.75±0.75	13.09±1.31	10.96±0.46	10.91±0.82	11.30±0.84	11.57±1.92	11.49±2.31
		NBA	10.00±1.36	10.16±1.31	9.43±0.46	9.25±0.82	9.13±0.84	10.08±1.93	10.10±2.31
		NWA	9.10±1.29	8.83±1.24	8.70±0.43	7.84±0.78	8.01±0.79	8.39±1.82	8.19±2.18
		MBW	1.35±0.08	1.23±0.08	1.39±0.03	1.42±0.05	1.39±0.05	1.28±0.11	1.56±0.14
		MWW	6.55±0.18	6.74±0.17	6.53±0.06	6.68±0.11	6.51±0.11	6.38±0.25	6.49±0.30
	Later parities	n	30	37	314	125	90	35	26
		TNB	10.42±0.98^{a}	12.04±0.77^{ab}	12.35±0.51^{ab}	12.03±0.68^{ab}	12.82±0.75^{b}	12.91±0.97^{b}	13.05±1.36^{c}
		NBA	9.70±1.12	11.02±0.87	11.19±0.59	10.76±0.78	11.16±0.86	11.34±1.09	11.55±1.54
		NWA	9.41±1.18	10.48±0.88	10.45±0.61	10.25±0.81	10.57±0.89	11.02±1.11	11.15±1.57
		MBW	1.65±0.08	1.53±0.06	1.45±0.05	1.49±0.06	1.56±0.07	1.44±0.08	1.57±0.12
		MWW	6.68±0.10	6.52±0.07	6.54±0.06	6.48±0.07	6.62±0.08	6.52±0.09	6.47±0.13
Landrace	First parity	n	11	15	136	82	60	22	10
		TNB	8.58±1.01	10.21±0.94	10.17±0.35	10.50±0.42	10.97±0.54	10.79±0.96	8.29±1.61
		NBA	7.56±1.13	8.29±1.06	8.71±0.39	9.45±0.47	9.05±0.60	9.94±1.09	8.09±1.81
		NWA	6.29±1.12	7.91±1.04	8.08±0.39	8.68±0.46	8.28±0.59	8.66±1.07	6.44±1.79
		MBW	1.55±0.08	1.62±0.08	1.57±0.03	1.52±0.52	1.51±0.05	1.60±0.08	1.61±0.13
		MWW	6.36±0.11	6.52±0.11	6.45±0.03	6.43±0.04	6.49±0.06	6.51±0.10	6.43±0.17
	Later parities	n	25	33	294	171	124	49	23
		TNB	9.60±0.69^{a}	10.29±0.60^{ab}	10.54±0.37^{b}	11.09±0.42^{b}	10.95±0.47^{b}	11.53±0.65^{b}	12.95±0.96^{c}
		NBA	9.00±0.81^{a}	9.07±0.69^{ab}	9.05±0.44^{ab}	9.78±0.50^{b}	9.81±0.56^{b}	10.63±0.76^{b}	12.10±1.06^{c}
		NWA	8.74±0.85^{a}	8.85±0.73^{a}	8.79±0.46^{a}	9.35±0.52^{ab}	9.70±0.58^{b}	10.46±0.80^{b}	11.55±1.17^{c}
		MBW	1.60±0.06	1.53±0.06	1.64±0.03	1.61±0.04	1.59±0.05	1.62±0.06	1.49±0.09
		MWW	6.71±0.10	6.50±0.08	5.56±0.06	6.57±0.06	6.56±0.07	6.54±0.09	6.51±0.12

[1]n: number of investigated litters, TNB: total number born, NBA: number born alive, NWA: number of piglets weaned alive, MBW: mean birth weight of the piglets, MWW: mean weight of piglets at weaning. MBW and MWW traits are expressed in kg.
[2]Means±SE represent least square means±standard error. Values in each row with differing superscripts are considered significantly different ($^{a, b, c}$ $P < 0.05$). Number of favorable alleles as accumulated alleles of the combined genotypes for porcine *FTH* g.9537834G, *FTH* g.9537855C, and *EPOR* g.70066473T.

DISCUSSION

In this study, we have elucidated the effects of *FTH* and *EPOR* polymorphisms on litter size traits in commercial Large White and Landrace pigs. Three SNPs loci of the porcine *FTH* g.9537834G > A, *FTH* g.9537855T > C, and *EPOR* g.70066473C > T polymorphisms were found to be segregating in these two pig populations. The porcine *FTH* g.9537834G, *FTH* g.9537855T, and *EPOR* g.70066473C were major alleles in these sows. The chi-square test showed that the genotype distributions of the porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C loci within Large White and Landrace sows deviated from the Hardy-Weinberg equilibrium specifications. Whereas the genotype distributions of the porcine *EPOR* g.70066473C > T locus within the pig populations agreed with the Hardy-Weinberg equilibrium specifications. The

results indicated that there are effects of selective pressures on some desirable production traits that are correlated with the porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C loci in these Large White and Landrace pig populations. However, the porcine *EPOR* g.70066473C > T locus was under homeostasis when accompanied by the effects of artificial selection in these pig populations. The porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C polymorphisms had significantly associated with the litter size traits in Large White and Landrace sows. The porcine *FTH* g.9537834G and *FTH* g.9537855C alleles seem to be positively correlated with litter size traits in the Landrace sows. Moreover, the porcine *EPOR* g.70066473C > T was significantly associated with the litter size traits in Large White and Landrace sows. The porcine *EPOR* g.70066473T allele seems to be a favorable allele for litter size traits in the pig populations.

The association of *FTH* polymorphism with reproduction traits in pigs has been scarcely studied. However, it has been demonstrated that a complete knockout of the *FTH* gene causes embryonic lethality in mice (Ferreira et al., 2000; Li et al., 2015). This evidence suggests that the *FTH* subunit plays an important role in embryogenesis (Li et al., 2015). Moreover, it has been reported that the *FTH* gene was expressed in the ovarian and endometrium tissues in various animal species (Gray et al., 2006; Yang et al., 2008; Chen et al., 2015). Similarly, the FTH protein was expressed in the corpus luteum during the pregnancy of rat (González-Fernández et al., 2008). In addition, the long-term selection to increase ovulation rate and decrease embryo mortality has been exhibited decreasing expression levels of the porcine *FTH* gene in the anterior pituitary (Bertani et al., 2004). Moreover, the porcine *FTH* polymorphisms are associated with the number of stillborn piglets (Pripwai and Mekchay, 2012). Thus, the *FTH* gene may be crucial for the embryo survival during pregnancy. The FTH induced an increase of gene expressions of interleukin-1β (IL-1β), IL-6, IL-12, and tumor necrosis factor-α (TNF-α), and stimulated macrophages to enhance the proliferation of peripheral blood mononuclear cells (PBMCs) (Ruscitti et al., 2020). These cytokine genes are involved in embryonic implantation and the maintenance of pregnancy (Blitek et al., 2012; Mathew et al., 2016). Moreover, the ferritin concentrations in early pregnancy were associated with placental development (Hindmarsh et al., 2000). This evidence indicates that the *FTH* gene may be necessary for the maintenance of pregnancy in animals. Although the functions of the porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C polymorphisms have not been characterized yet, the 3′-UTR of the porcine *FTH* gene was aligned with the 3′-UTR of the human *FTH* gene (www.genome.jp/tools-bin/clustalw, accessed on 30 September 2021) and the putative microRNA targets on the 3′-UTR of the human *FTH* gene were predicted using the TargetScanHuman v8.0 database (www.targetscan.org, accessed on 30 September 2021) due to the limited number of available porcine miRNA in public databases. The 3′-UTR of the porcine *FTH* gene showed homology with the 3′-UTR of the human *FTH* gene (Figures 3A and 3B). Interestingly, these two polymorphisms are located in the 3′-UTR of the porcine *FTH* gene and these SNPs are positioned within the binding site of the putative seed regions of the hsa-miR-6890-5p, hsa-miR-3675-5p, hsa-miR-9500, hsa-miR-7113-5p, and hsa-miR-6753-5p as indicated in Figures 3A and 3B. It might lead to non-complementarity between

these miRNAs and the SNPs in 3′-UTR of the *FTH* mRNA pairing (Figures 3C and 3D). These miRNAs might disrupt *FTH* expression, resulting in that the porcine *FTH* polymorphisms had effects on litter size traits in pigs. Numerous studies have demonstrated that several miRNAs bind with 3′-UTR of the *FTH* mRNA and revealed down-related its expression, resulting in the alterations of ferritin functioning in various cell types (Davis and Clarke, 2013; Chan et al., 2018). Moreover, it has been reported that SNPs within the binding site of miRNA can affect miRNA-induced genetic repression (An et al., 2015). This result is consistent with previous studies, which indicated that a SNP (rs55618224) at the 3′-UTR of porcine cell division cycle 42 (*CDC42*) gene disrupted the binding site for miR-18a and had significant effects on litter size in pigs (Liu et al., 2019). Similarly, SNPs in caprine prolactin receptor (*PRLR*) and KIT ligand (*KITLG*) genes influenced the binding site of bta-miR-302a, chi-miR-204-5p, and chi-miR-211 and had significant effects on litter size in goats (An et al., 2015; An et al., 2016). Moreover, the porcine *FTH* g.9537834G > A and *FTH* g.9537855T > C polymorphisms may be in the close linkage of disequilibrium with the causative SNPs that are known to have a positive effect on litter size traits in pigs.

Figure 3 Consensus of nucleotide sequences of the 3′-UTR of porcine *FTH* mRNA (GenBank accession no. NM_213975) and human *FTH* mRNA (NM_002032), and prediction of miRNA targets of the porcine *FTH* gene at (A) g.9537834G > A and (B) g.9537855T > C loci. The SNPs of (C) porcine *FTH* g.9537834G > A and (D) g.9537855T > C are located within the bind sites of putative seed regions of miRNA targets. The SNPs positions are indicated by capital bold letters. The putative seed-pair region is indicated by shaded areas. | = complementary base pairing.

The association of *EPOR* polymorphism with litter size traits has been identified in pigs (Vallet et al., 2005; Zhang et al., 2011a; 2011b). The porcine *EPOR* polymorphism was associated with uterine capacity and litter size in pigs (Vallet et al., 2005). There was an increased number born alive (about 2.7 piglets) in CT genotype when compared with the CC genotype ($P < 0.05$). This result is consistent with our present study that showed the positive effects of the porcine *EPOR* g.70066473T allele on the litter size traits. Conversely, there was no association of the porcine *EPOR* g.2373 C > T locus with litter size traits in the Chinese pig population (Zhang et al., 2011b). This contrasting result may be caused by the different genetic backgrounds and population structures of the pig breeds. *EPOR* is directly essential for red blood cell production (Dev et al., 2013). The previous studies revealed that *EPOR* is involved in fetal survival by promoting the growth of red blood cells and uterine capacity in sows (Christenson, 1993; Vallet et al., 2002). Fetal erythropoiesis influences uterine capacity during early pregnancy (Pearson et al., 1998). The maturation of the fetal blood supply, an increase in red blood cell number, and maturity may contribute to the greater uterine capacity (Vallet et al., 2003). This evidence indicates that the fetal erythropoiesis during the pregnancy has an effect on the improvement of uterine capacity and in litter size traits of mammals. Although mRNA and protein expression of *EPOR* in reproductive organs of pigs have not been studied, protein levels of EPO and EPOR have been evaluated in guinea pigs. The upregulation of these proteins can promote erythropoiesis in response to systemic hypoxia (Semenza, 2009; Elias et al., 2017). In *EPO* and *EPOR* knockout mice, death occurs on embryonal days 11–13.5, owing to the failure of definitive erythropoiesis in the liver (Baumann and Dragon, 2005). The porcine *EPOR* g.70066473C > T locus is involved in the binding site for GATA-1, Sp1, and CCACC-binding protein (CBP). These binding site proteins were implicated in transcriptional control in erythroid cells, which may be the reason for affecting the fetal erythropoiesis and litter size traits (Ohneda and Yamamoto, 2002; Vallet et al., 2005). Thus, the porcine *EPOR* g.70066473C > T may affect the transcription and expression of the *EPOR* gene, resulting in this polymorphism having effects on litter size traits in pigs. Moreover, it might be associated with the causative SNPs that are known to have a positive effect on litter size traits in pigs.

Although the relationship between the porcine *FTH* and *EPOR* genes with litter size traits in pigs has been scarce reported, it has been demonstrated that the two critical components of iron and erythropoietin are required for erythrocyte production in erythropoiesis (Ganz, 2018). The absence or deficiency of iron or erythropoietin leads to reduced erythropoiesis and decreased embryonic survival during embryogenesis and pregnancy (Ganz, 2018). Additionally, both *FTH* and *EPOR* are involved in the binding site for GATA-1 that is implicated in transcriptional control in erythroid cells and might be affected the erythropoiesis (Vallet et al., 2005; Zolea et al., 2017). These pieces of information suggested that the ferritin and erythropoietin receptor seem to be strongly interrelated by their function in erythropoiesis and are associated with embryogenesis and embryo survival in mammalian species. Therefore, it was explored whether there is a relationship between the porcine *FTH* and *EPOR* genes in terms of their accumulated favorable alleles

with the litter size traits in pigs. There is an important procedure to accumulate identified genes from multiple parents into a superior genotype of the breeding stock (pyramiding approach) to establish an elite breeding stock with high prolificacy traits in pigs. Therefore, analysis of the effects of accumulated favorable alleles on litter size traits is required (Norseeda et al., 2021d). In this study, the association of accumulated favorable alleles of the porcine *FTH* g.9537834G > A, *FTH* g.9537855T > C, and *EPOR* g.70066473C > T loci had effects on litter size traits in pigs. The accumulated favorable alleles of the porcine *FTH* g.9537834G, *FTH* g.9537855C, and *EPOR* g.70066473T (GGCCTT) were indicated as a favorable genotype with the highest values for the TNB, NBA, and NWA traits. On the other hand, the *FTH* g.9537834A, *FTH* g.9537855T, and *EPOR* g.70066473C (AATTCC) were indicated as an unfavorable genotype with the lowest values for these traits. Thus, the accumulated favorable alleles (GGCCTT) may be used as potential markers for selecting individuals with higher litter size traits. This evidence indicates that there are strong additive effects of the accumulated favorable alleles of porcine *FTH* and *EPOR* genes on the litter size traits. The association of the porcine *FTH* and *EPOR* genes with litter size traits may be implicated in the angiogenic and vasculogenic processes of embryos. These two processes are involved in the blood vessel development in the placenta (Demir et al., 2010). It has been reported that the ferritin-mediated regulation of angiogenesis represents ferritin blocks the anti-angiogenic protein activity that promotes adhesion and survival signaling in endothelial cells (Coffman et al., 2009; Tesfay et al., 2012). Moreover, *EPOR* and its ligand (*EPO*) are expressed in the vasculature during embryogenesis. Deletion of *EPO* and/or *EPOR* in mouse embryos exhibit angiogenic defects, cardiac failure, and leads to the death of the embryonic lethal phenotype (Wu et al., 1999; Makita et al., 2001; Kertesz et al., 2004). Additionally, *EPOR* promotes angiogenesis through upregulation of vascular endothelial growth factor (VEGF) and VEGF receptor system by enhancing neovascularization and recruiting endothelial progenitor cells (Nakano et al., 2007). These findings indicated that the polymorphisms of the *FTH* and *EPOR* genes may contribute to embryonic implantation, placenta development, and fetal survival. Therefore, it could be consolidated that the polymorphisms of the porcine *FTH* and *EPOR* genes are associated with the litter size traits of pigs. Further studies need to focus on the molecular functions of the *FTH* and *EPOR* as well as its ligand genes in embryogenesis and placental development of pigs. Moreover, two variants of SNPs in 3′-UTR of the porcine *FTH* gene are required to elucidate whether it has affected the binding affinity of targeted miRNA regulation in pigs.

CONCLUSION

In the present study, we have examined the effects of polymorphisms of the porcine *FTH* and *EPOR* genes as well as their accumulated favorable allele effects on litter size traits in Large White and Landrace pig breeds. The polymorphisms of the porcine *FTH* and *EPOR* genes revealed an association with litter size traits (TNB, NBA, and NWA). Furthermore, the accumulation of favorable alleles of porcine *FTH* and *EPOR* genes showed an association

with litter size traits (TNB, NBA, and NWA). These findings suggest that porcine *FTH* and *EPOR* genes may contribute to the reproductive processes of pigs with regards to litter size and confirm the importance of these two genes as functional candidate genes for improving litter size in pigs.

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AUTHOR CONTRIBUTIONS

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