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Association of *IGF1R* Polymorphisms with Growth Traits and its Expression Profiles in Different Pig Breeds

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ABSTRACT

Insulin-like growth factor 1 receptor (IGF1R) is one component of insulin-like growth factor system, which has biological functions of growth traits. The aim of this study is to investigate the entire exons of *IGF1R* in the three commercial pig breeds, Duroc, Yorkshire and Landrace, to identify novel single nucleotide polymorphisms and their correlation with growth traits. One novel SNP (c.3678C>T) in the exon 20 was detected. This SNP caused the change of amino acid (Ser1217Phe), a portion of cytoplasmic tyrosine kinase domain of IGF1R. At the c.3678C>T site, three genotypes were significantly associated with average daily gain at different growth stage in Yorkshire and Landrace breeds. Meanwhile, we identified the differently expressed pattern of *IGF1R* in muscle of Yorkshire and Jinhua pigs at different growth stages. Our results provide useful information on understanding the effect of porcine *IGF1R* gene on growth. The novel *IGF1R* polymorphism may be useful as molecular markers in pig selection but future studies are required.

Key words: *IGF1R*, SNP, Pig, Average daily gain, Body weight, Gene expression.

INTRODUCTION

The importance of average daily gain (ADG) and body weight (BW) in pig breeding programs and in meat production industry motivate researchers and animal breeders to dissect the genetic architecture behind these growth traits (Ji *et al*., 2019). Single nucleotide polymorphisms (SNPs) in genomic regions, which accounting for significant portions of phenotypic variations for economically important traits, have been identified and implemented in marker assisted selection (Andersson *et al*., 1994; Blaj *et al*., 2018). A genome-wide scan through selection signatures in Chinese indigenous and commercial pig breeds reveals that *IGF1R* gene was associated with growth and development (Yang *et al*., 2014; Zhang *et al*., 2018).

Porcine *IGF1R* gene has been mapped to the chromosome 1, which contains 21 exons and 20 introns. IGF1R protein contains receptor L domain, furin-like cysteine rich region, fibronectin type 3 domain and the cytoplasmic tyrosine kinase domain, which is an unstable in domestic animals (Sahoo *et al*., 2019). Several reports indicate that numerous mutations in *IGF1R* affect late prenatal and early postnatal growth restriction, perinatal growth velocity and diminutive body size (Harris *et al*., 2014). *IGF1R* null mice die at birth of respiratory failure and exhibit only 45% of the body weight of their wide type littermates (Liu *et al*., 1993). The potential association between the polymorphisms in the *IGF1R* locus and traits with economic importance have been investigated (e.g., BW, ADG) in different species of animals, including cow (Leyva-Corona *et al*., 2018), buffalo (El-Magd *et al*., 2013) and chicken (Lei *et al*., 2008). In pigs, the role of *IGF1R* in the regulation of the ADG and BW is still intelligible.

The objectives of this study were to examine the associations of SNPs in *IGF1R* gene with growth traits in three pig populations, Duroc, Yorkshire and Landrace. Meanwhile, the expression pattern of *IGF1R* was detected in Yorkshire and Jinhua pig breeds.

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MATERIALS AND METHODS

Animal care

The experimental procedure was approved by the Institutional Animal Care and Use Committee at Hebei University of Engineering (AEEI-16015).

Animals and Trait Measurements

The experimental populations included 406 pigs, specifically 62 Duroc (male = 22, female = 40), 154 Landrace (male = 40, female = 114) and 190 Yorkshire (male = 60, female = 130) pigs. The pigs maintained at three different farms belonged to Yunnan Honghe Bangge Animal Husbandry Co., Ltd. under the same housing and feeding system. The feeding system contained the grower stage from 30kg to 50kg of body weight and the finisher stage from 50kg to 100kg of body weight. Body weights at 70 d of age as well as the age when the pigs reached the target weight were recorded. The target weight contained 30kg, 50kg and 100kg. We also measured the ADG from 30kg to 50kg, from 50kg to 100kg, from 30kg to 100kg and the backfat thickness

when the body weight was 100kg. Longissimus muscle tissues were collected from six Yorkshire and six Jinhua pigs at each 30, 90 and 180 days of age. A total of 36 pigs were sampled. The samples were taken immediately after euthanasia, frozen in liquid nitrogen and stored at –80°C until use.

DNA extraction and polymorphism analysis

Genomic DNA was extracted from the ear tissues using a standard phenol-chloroform method then quantified using a NanoDrop 2000 spectrophotometer. *IGF1R* polymorphisms in all exons were screened. The PCR primers were designed basing on the sequence of pig *IGF1R* (Gene Bank accession. NC_010443.5) (Table 1). PCR amplifications were carried out in 20 µL reaction volumes containing at the following final concentrations: 50 ng of template DNA, 400 µM of dNTPs, 0.25 µM of primer and 1 unit of Taq polymerase. The PCR protocol consisted of an initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at annealing temperature of each pair primer for 30 s, extending at 72°C for 30 s and then with a final extension at 72°C for 10 min. The PCR products were directly sequenced. Sequences were aligned for identification of mutations.

Based on the sequencing, a novel polymorphism was identified in the exon 20: c.3678C>T, which was genotyped by the Created Restriction Site PCR (CRS-PCR). The mismatched base in the primer enabled the application of restriction enzymes to discriminate the sequence variations. Meanwhile, the reported polymorphism in intron 9 was also genotyped using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), which was named as allele A (379 bp) or allele B (235bp and 144bp) (Kopecny *et al*. 2002). *Sac*II and *Taq*I were two kinds of endonucleases detecting the SNP localized in intron 9 and exon 20, respectively. After PCR, 5 µL of PCR product, 0.2 µL of endonuclease, 1 µL 10×buffer and 3.8 µL ddH_2O were mixed and placed in a 37°C or 65°C thermostatic water bath overnight. The enzyme-digest products were subsequently identified by electrophoresis on 1.5% agarose gels and genotyped according to the fragment length. The details of the obtained DNA fragment after endonuclease digestion are presented in Table 2.

RNA isolation and Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from porcine longissimus muscle tissues using TRIzol reagent. The quality of RNA was evaluated using a NanoDrop 2000 spectrophotometer. Only samples with an A260/A280 ratio of 1.8-2.0 and an A260/A230 ratio greater than 2.0 were used for subsequent analysis. Reverse transcription of RNA to cDNA was performed using an Improm-II Reverse Transcriptase kit following the manufacturer's instructions. qPCR was performed using SYBR Green Universal Master mix, following the manufacturer's recommendations in a CFX96 Real-Time system. The PCR program was 95°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a melting curve analysis (65–95°C) in the last cycle to evaluate amplification specificity. Beta-actin (ACTB) was used as an endogenous control. The primer sequences are shown in Table 3.

Table 1: PCR amplification primers for *IGF1R.*

IGF1R	Sequence of primers (5′→3′)		Length (bp)
Exon1	F: CGCCGCCTTTGGAGTATT	R: AAACACCGAGGCCCTTCC	467
Exon2	F: CTAGTGGGACAGGAATGGC	R: AGAGGAAGGAGGGTGAGG	895
Exon3	F: CCTGCCTTGATGAAACTCC	R: ATTGCCCATTGCTCCTTG	687
Exon4	F: CAGTTTCTCCCTCTTGG	R: GGGAAATTCAAAGGAGG	631
Exon5	F: TCAGGGTGAGGGTGTTA	R: CTCCCACTTTGCTGACCAT	565
Exon6	F: GAGCATCCTTCCGTTTG	R: TCATCAAGAAGCCCTCC	532
Exon7	F: TGCCACCAAGTGTCCTA	R: TGCTTCCCACCAAACAG	522
Exon8	F: ATCAGCGTTTTCAGTCC	R: ATGCCAACCAGACAGAT	618
Exon9-10	F: TTTCAGCGTCCAACACC	R: GACCGACAAACCACCAG	743
Exon11	F: TGAGGTTTGCCGTGGAG	R: TCATTGCCCTGGGTGTC	580
Exon12	F: GACCTCTAGCCTGGGAATC	R: TCAGGGAACTCCTCACC	502
Exon13	F: CACAGTTCCTGGAGGGC	R: CCAGCATCCCTGACGAG	653
Exon14	F: TGACTGGGCAGTAAAGA	R: ATGGCTGTGGTGTAGGC	446
Exon15	F: ACAATACCCTCCCTTCC	R: AGGCCAAAGCTACCTCA	340
Exon16	F: GGATGACCTCCTGGCTCT	R: ACCCTGGATGATGTGGC	615
Exon17	F: CTTATGGGTTTGCGACTC	R: AGTCAAGGAACACGCTCT	440
Exon18	F: GTAATGTCCTTTCCCTCC	R: CTCTTGCCCACTACCTG	774
Exon19	F: TCCCTGCTGGTTTCTGC	R: AGGAACACGCCAACGAC	465
Exon20	F: CTGGCTCCTTGGCTGAC	R: CGGCAAACCGCAAACCT	437
Exon21	F: CAGTGGTGGATGTAAGTCAG	R: TTAAGGGTTTGGGTTGG	790

Table 2: The details used to identify the polymorphisms in the *IGF1R* locus.

SNP	Primers Sequence (5′→3′)	Endonuclease	Obtained Alleles
Intron9 (A/B)	F: AGCTATCTCTACCGGCATAA	*Sac*II	A-379bp
	R: TCTCGAAGACCTTGCGGTACT		B-235, 144bp
c.3678C>T	F: AGCAGCCATACCAGGGCTTGTCG	*Taq*I	T-235
	R: CGGCAAACCGCAAACCT		C-200, 35bp

Table 3: RT-qPCR primers for *IGF1R* and *ACTB*.

Gene	Primers Sequence(5′→3′)	Length (bp)	Annealing temperature (°C)
IGF1R	F: CAACCTCCGGCCTTTTACTTT	162	60
	R: CAGGAATGTCATCTGCTCCTTCT		
ACTB	F: CCAGCACCATGAAGATCAAGATC	102	60
	R: ACATCTGCTGGAAGGTGGACA		

Table 4: The genotypic and allelic frequencies of *IGF1R* gene SNPs in different pig breeds.

SNP	Breed[1)]	Genotype	GF[2)]	Allele	AF[3)]	χ2	He	Ne	PIC
Inton9 (A/B)	D	AA	1	A	1	-	0	1	0
		AB	0						
		BB	0	B	0				
	L	AA	1	A	1	-	0	1	0
		AB	0						
		BB	0	B	0				
	Y	AA	1	A	1	-	0	1	0
		AB	0						
		BB	0	B	0				
c.3678C>T	D	CC	0.0323	C	0.16	0.13	0.27	1.3709	0.2340
		CT	0.2580						
		TT	0.7097	T	0.84				
	L	CC	0.2143	C	0.28	70.7**	0.40	1.6737	0.3215
		CT	0.1299						
		TT	0.6558	T	0.72				
	Y	CC	0.0579	C	0.09	61.8**	0.17	1.2070	0.1568
		CT	0.0737						
		TT	0.8684	T	0.91				

[1)] D: Duroc, L: Landrace, Y: Yorkshire. [2)] GF: Genotypic frequency. [3)]AF: Allele frequency.

Statistical analysis

Statistical analyses were performed using the GLM (General Linear Model) procedure of the SAS (v9.4; SAS Institute, Cary, NC). The LSM (Least Squares Means) method was used to determine the statistical significance between groups. The linear model was: $Y_{ijklm} = \mu + A_i + B_j + C_k + R_l + e_{ijklm}$, where Y_{ijklm} is the observation of the trait; μ is the population mean; A_i is the fixed effect of the experimental farms, B_j is the fixed effect of breed, C_k is the fixed effect of sex, R_1 is the fixed effect of the haplotype and the e_{ijklm} is the random residual. The analyzed animals were not related. The results are presented as mean ± standard error (SE). Significant and extreme differences were set at $P < 0.05$ and $P < 0.01$, respectively.

Gene frequencies were determined by direct counting. He (gene heterozygosity), Ne (effective number of alleles) and PIC (polymorphism information content) were calculated according to previous report (Kumar *et al*. 2020), respectively. The formulas were

$$He = 1 - \sum_{i=1}^{n} (P_i)^2,$$

$$Ne = \frac{1}{\sum_{i}^{n} (P_i)^2} \text{ and}$$

$$PIC = 1 - \sum_{i=1}^{n} (P_i)^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Where

P_i and P_j are the frequency of the i and j allele and n is the number of allele.

In terms of the RT-qPCR, all samples were analyzed in triplicate and normalized to *ACTB* mRNA levels, with values expressed as means ± standard deviation (SD). Significance was determined using a Student's unpaired t-test in SAS (v.9.4; SAS Institute, Cary, NC) with a significant

level of $P < 0.05$.

RESULTS AND DISCUSSION

A total of 21 exons of *IGF1R* gene were sequenced and only a novel polymorphism in exon 20 (c.3678C>T) were detected. PCR product digestion by endonuclease *Taq*I generated fragments with lengths of 200bp and 35bp for genotype CC, 235bp for genotype TT and 235bp, 200bp and 35bp for genotype CT (Table 2, Fig 1). Conflicting to a previous study reported by Harumi *et al*. (2001), who identified 12 silent sequence variants in coding regions based on RT-PCR derived *IGF1R* sequence in 12 unrelated pigs. The novel SNP in the exon 20 was a missense mutation and located in the cytoplasmic tyrosine kinase domain of IGF1R protein. The mutations in the kinase domain can cause conformational changes consequently affect the binding capability (Gately *et al*., 2015). Hence, the polymorphism in exon 20 (c.3678C>T) resulting in the amino acid changing might have serious consequences for ligand binding, which needed to be further verified.

At the c.3678C>T site, the frequency of the T allele was greater than that of the C allele in the three pig breeds. The frequency of the TT genotype was higher than the other two genotypes. According to the $\chi 2$ test statistics, the SNP c.3678C>T in Duroc met Hardy-Weinberg equilibrium ($P > 0.05$), while which in Landrace and Yorkshire breed deviated from the Hardy-Weinberg equilibrium ($P < 0.01$). The He of the mutant loci was 0.27, 0.40 and 0.17 in different pig breeds, respectively (Table 4). It was likely that allele frequencies at the QTL is different among breeds and thereby influence genetic and phenotypic variance. The association analysis of *IGFR1* gene genotypes and recorded growth traits within the three different population were analyzed (Table 5). In Landrace breed, animals with the genotype CC had significantly higher ADG of 30-50kg than those with genotypes TT ($P < 0.05$). In Yorkshire breed, genotype CC showed differences in ADG of 30-50kg compared to genotypes TT ($P < 0.05$) and also in the ADG of 50-100kg ($P < 0.01$). The CC individuals were characterized by great ADG of 30-50kg and ADG of 50-100kg. In Duroc breed, this SNP had no significant correlation with the recorded growth. Moreover, differences in growth traits between breeds have been reported (Lents *et al*., 2016; Tyra *et al*., 2019).

In the present study, we also screened the SNP in intron 9 in the Duroc, Landrace and Yorkshire breeds. However, only the AA genotype was identified (Table 4), which was consistent with the previous study that the European pigs principally carried allele A and the pigs with AA genotype exhibited greater body weights (Kopecny *et al*., 2002; Wang *et al*., 2006).

Meanwhile, *IGF1R* has different influences at various stages of growth and its expression in regulated depending on developmental and functional status (Cheng *et al*., 2016; Yang *et al*., 2019). We detected the differently expressed pattern of *IGF1R* in muscle of Yorkshire and Jinhua pigs at different growth stages (Fig 2). In Jinhua pigs, *IGF1R* had significantly higher expression at 30-day than at 90-day and 180-day. Compared with Jinhua pigs, *IGF1R* showed lower expression at 30-day but higher expression level at 90-day in Yorkshire pigs ($P < 0.05$). There were no significant differences at 180-day. We speculate that the dramatic difference in longissimus dorsi muscle growth between the

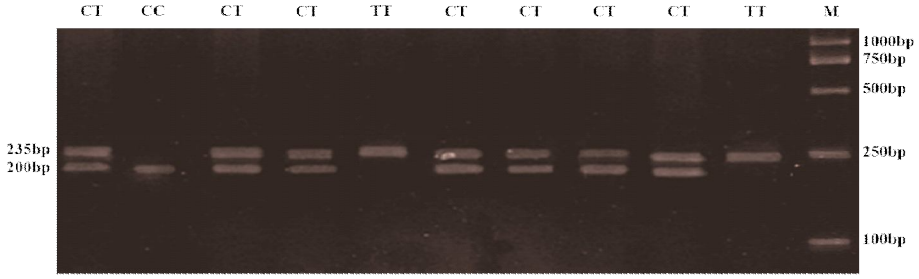

Fig 1: PCR-RFLP patterns of the novel SNP in exon 20.

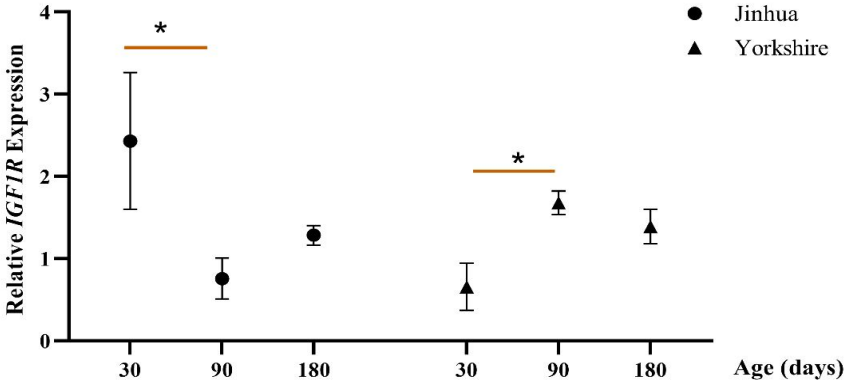

Fig 2: Expression of *IGF1R* in muscle of Yorkshire and Jinhua pigs. *$P < 0.05$.

Table 5: Least squares mean ± SE for growth traits among genotypes of the novel SNP in the *IGF1R* gene.

Growth traits	c.3678C>T in Landrace			c.3678C>T in Yorkshire			c.3678C>T in Duroc		
	CC	CT	TT	CC	CT	TT	CC	CT	TT
BW70 (kg)	28.3±1.09	27.3±0.90	26.2±0.63	25.4±2.48	28.3±0.60	27.0±0.50	28.4±2.48	28.3±0.60	27.0±0.50
D30 (days)	72.3±1.29	72.3±4.15	74.0±1.07	75.5±3.34	72.2±3.96	74.0±1.17	72.6±4.54	70.3±1.21	72.3±0.68
D50 (days)	101.1±1.60	100.9±5.17	100.5±1.34	99.9±4.37	107.2±5.18	100.8±1.53	97.7±5.07	99.0±1.35	99.3±0.76
D100 (days)	161.4±2.09	156.8±6.76	159.1±1.75	159.1±5.17	159.2±6.13	161.9±1.81	155.5±6.81	159.3±1.82	159.7±1.02
ADG of 30-50kg (g)	766.9±22.05^{a}	693.8±85.32^{ab}	701.2±26.42^{b}	843.8±71.72^{a}	778.5±25.39^{ab}	628.1±85.01^{b}	805.1±123.32	717.4±32.93	758.5±18.41
ADG of 50-100kg	846.7±20.82	898.9±67.23	863.2±17.37	983.3±52.36^{A}	859.1±44.18^{AB}	833.2±15.49^{B}	872.3±80.46	844.8±21.48	842.5±12.01
ADG of 30-100kg	793.0±14.46	830.8±46.71	828.1±12.07	843.1±34.25	807.0±40.59	802.5±12.01	847.8±59.94	794.4±16.01	809.8±8.95
BT (100kg) (mm)	8.9±0.29	9.0±0.94	9.1±0.24	8.9±0.63	8.8±0.75	9.2±0.22	10.1±1.08	9.5±0.29	9.2±0.16

$^{A, B}$ or $^{a, b}$Among genotypes within each SNP for each trait, means without a common superscript differ ($P < 0.01$) or ($P < 0.05$).

two breeds may be partially regulated by *IGF1R* at early stage. It is consistent with the difference in longissimus dorsi muscle area observed after but not before day 90 in Jinhua and Landrace pigs, the latter of which are similar in growth to Landrace pigs (Wu *et al*., 2013).

CONCLUSION

Overall, a novel SNP (c.3678G>A) was identified in the exon 20 of porcine *IGF1R* gene that encoded a portion of cytoplasmic tyrosine kinase domain and caused the amino acid changing. The association analysis showed that the SNP c.3678G>A had significant impact on ADG. Effects of CC, CT and TT genotypes on the ADG at early and late growth stages were different in the three commercial pig breeds. The novel *IGF1R* polymorphism may be useful as molecular markers in pig selection but needed to be future studied. Additionally, the *IGF1R* expression had different profiles in Yorkshire and Jinhua pigs. Despite the lack of the association of the novel SNP at exon 20 along with the *IGF1R* expression pattern, our results provide a basis for further studies on the genetic influence of *IGF1R* gene on pig growth and development.

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