Characterization of Phosphoinositide-3-kinase, Class 3 (PIK3C3) Gene and Association Tests with Quantitative Traits in Pigs

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ABSTRACT : This study deals with the characterization of porcine *PIK3C3* and association tests with quantitative traits. *PIK3C3* belongs to the class 3 PI3Ks that participate in the regulation of hepatic glucose output, glycogen synthase, and antilipolysis in typical insulin target cells such as those in the such as liver, muscle system, and fat. On the analysis of full-length mRNA sequence, the length of the *PIK3C3* CDS was recorded as 2,664 bps. As well, nucleotide and amino acid identities between human and pig subjects were 92% and 99%, respectively. Five SNPs were detected over 5 exons. We performed genotyping by using a SNP C2604T on exon24 for 145 F_2 animals (from a cross between Korean native boars and Landrace sows) by PCR-RFLP analysis with *Hpy*8I used to investigate the relationship between growth and fat depot traits. In the total association analysis, which doesn't consider transmission disequilibrium, the SNP showed a significant effect ($p<0.05$) on body weight and carcass fat at 30 weeks of age as well as a highly significant effect ($p<0.01$) on back fat. In an additional sib-pair analysis, *C* allele still showed positive and significant effects ($p<0.05$) on back fat thickness and carcass fat. Moreover, the effects of *C* allele on the means of within-family components for carcass fat and back fat were estimated as 2.76 kg and 5.07 mm, respectively. As a result, the SNP of porcine *PIK3C3* discovered in this study could be utilized as a possible genetic marker for the selection of pigs that possess low levels of back fat and carcass fat at the slaughter weight. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 12 : 1701-1707)***

Key Words : *PIK3C3*, SNPs, PCR-RFLP, Sib-pair Analysis, Back Fat, Carcass Fat

INTRODUCTION

Quantitative trait loci (QTL) indicate the location of genes on chromosomes that affect important economic traits. Many experiments have been performed and have succeeded in finding QTLs associated with growth, fat depot and reproduction in domestic animals (Rohrer and Keele, 1998; Bidanel et al., 2001; Brym et al., 2004; Li et al., 2004; Zeng et al., 2005).

In pigs, QTL analyses related to growth and carcass traits have been performed for 18 autosomes and the X chromosome (Knott et al., 1998; Wang et al., 1998; Harlizius et al., 2000; Ovilo et al., 2002; Nagamine et al., 2003; Gaboreanu et al., 2004; Kim et al., 2004). Evidences for QTL as related to back fat thickness (BF) and intramuscular fat contents (IMF) have been reported on porcine chromosome 6 (SSC6). In fact, de Koning et al. (1999) and Ovilo et al. (2002) detected a QTL associated with BF and IMF on porcine chromosome 6q. In addition, Grindflek et al. (2001) reported that the genes for IMF are located between the markers *SW1823* and *S0003*. As well, heart fatty acid-binding protein (*FABP3*) gene involved in fatty acid oxidation was reported to be associated with BF and IMF, with this gene assigned to SSC6q in pigs (Gerbens et al., 2000, 2001; Urban et al., 2002).

Kim et al. (2005) recently reported on the chromosomal positions of the porcine phosphoinositide-3-kinase, class 3 (*PIK3C3*) gene by a PCR analysis of a porcine×rodent somatic cell hybrid panel (Yerle et al., 1996) and a radiation hybrid panel of the porcine genome (Yerle et al., 1998). *PIK3C3* is more closely linked to *S0228* on chromosome 6q22-23 and the location of this gene was inferred to be between the markers *SW1823* and *S0003*. PIK3C3 (phosphoinositide-3-kinase, class 3) participates in mitogenesis, glucose transport, regulation of hepatic glucose output, glycogen synthase, and antilipolysis in typical insulin target cells such as those in the liver, muscle system, and fat (Shepherd et al., 1998; Czech and Corvera, 1999). Finally, this protein is essential for mammalian development, including its role in performing many specific cellular functions as well as for metabolic activity of insulin. Therefore, *PIK3C3* would appear to be a positional and functional candidate gene related to fat depot trait in domestic pigs.

The aims of this study are to characterize porcine *PIK3C3*, including identification of its full-length mRNA, detection of single nucleotide polymorphisms (SNPs), phylogenic analysis etc. and the fulfillment of an association study between SNP detected and quantitative traits.

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Table 1. Primer sequences used for amplifying porcine *PIK3C3* in this study

Analysis methods	Primer names	Primer sequences
CDS analysis	PIK3C3-F	5'-ATGGGGGAAGCAGAGAAGTTTCACTACATC-3'
	PIK3C3-R	5'-AGATTACAATGTTCTCTTCCTTGCTCGTTAGTC-3'
5' RACE-PCR	RACE-5'-1	5'-CATTGGGTCTTCTAGAACGGC-3'
	RACE-5'-2	5'-GCTGCACGTTGATATCCAGG-3'
3`RACE-PCR	RACE-3'-1	5'-TCTGTTTGCTGCGGTGGTAG-3
	RACE-3'-2	5'-GACTAACGAGCAAGGAAGAG-3'
PCR-RFLP	E24-F	5'-ATTTCGTCTAGACCTGTCCG-3'
	E24-R	5'-TGAATCTGTTCTACCACCGC-3'

MATERIALS AND METHODS

Animals

Skeletal muscle tissues from a Large White and a Korean native pig (KNP) were used for developing mRNA sequence analyses such as the cloning of full-length cDNA and detecting SNPs. In order to confirm the presence and to estimate the frequency of the SNP applied for association analyses, 20 individuals from each of four breeds (Landrace, Large White, Duroc, and KNP) were genotyped. One hundred and forty-five F_2 animals produced from a cross between 5 Korean native boars and 11 Landrace sows were analyzed for association tests. These animals included complete records of pedigree, BF, carcass fat content (CF), IMF and body weights at birth as well as 3, 5, 12, 20, and 30 weeks of age.

Nucleic acid isolation and cDNA synthesis

Genomic DNA was extracted from the whole blood with the Wizard Genomic DNA Purification Kit (Promega, USA). Total RNA was isolated from the skeletal muscle tissues using Trizol (Gibco BRL, USA), while the mRNA was purified by the Oligotex mRNA Isolation Kit (Quagen, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using the Smart™ Rapid Amplification of cDNA Ends (RACE) Kit (Clontech, USA).

Amplification and sequence analysis of porcine *PIK3C3*

The porcine specific primers necessary to amplify nearly full coding sequences (CDS) were designed from human (GenBank accession no. NM_002647) and mouse (GenBank accession no. NM_181414) mRNA sequences (Table 1). RT-PCR was carried out with a total volume of 25 μl containing 25 ng of the 1st strand cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM $MgCl_2$, 0.2 μM of each primer, 150 μM of each dNTP and 1.5 units of *Taq* polymerase (Takara, Japan). A 5-min denaturation at 94°C was followed by 35 cycles (30 s at 94°C, 60 s at 64°C and 90 s at 72°C) and a final extension of 10 min at 72°C on a PTC-200 Programmable Thermal Controller (MJ Research, Inc., USA). The PCR product was cloned using the TOPO TA Cloning Kit (Invitrogen, USA). Plasmid DNA was purified using the Minipreps DNA Purification System (Promega, USA) and sequenced on an Applied Biosystems 3,700 DNA sequencer (PE Applied Biosystems, USA). The sequences determined were then aligned using the Clustal X version 1.83 (Thompson et al., 1997) to detect SNPs.

Cloning of full-length cDNA by RACE-PCR

RACE-PCRs were used for obtaining full-length cDNA sequences with the 1st strand cDNA prepared by the Smart™ RACE cDNA Amplification Kit. The porcine *PIK3C3* specific primers were designed on the basis of the nucleotide sequences as determined by RT-PCR (Table 1). A 1st RACE-PCR was carried out with a total volume of 50 μl, containing 50 ng of the 1st strand cDNA, 50 mM of KCl, 10 mM of Tris-HCl (pH 8.3), 0.5 mM of $MgCl_2$, 0.2 μM of PIK5-1 or PIK3-1 primer, 5 μl of 10× Universal Primer A Mix (Clontech, USA), 150 μM of each dNTP and 2.5 unit of *Taq* polymerase (Takara, Japan). A nested RACE-PCR was carried out with a total volume of 50 μl, containing 3 μl of the 1st RACE-PCR product, 50 mM of KCl, 10 mM of Tris-HCl (pH 8.3), 0.5 mM of $MgCl_2$, 0.2 μM of PIK5-2 or PIK3-2 primer, 2 μl of Nested Universal Primer A (Clontech, USA), 150 μM of each dNTP and 2.5 units of *Taq* polymerase (Takara, Japan). The RACE PCR products were directly sequenced on the Applied Biosystems 3,700 DNA sequencer (PE Applied Biosystems, USA).

Restriction fragment length polymorphism (RFLP) analysis

A primer set was designed for genotyping the SNP C2604T (Table 1). A PCR reaction was carried out with a total volume of 25 μl, containing 25 ng of genomic DNA, 50 mM of KCl, 10 mM of Tris-HCl (pH 8.3), 0.5 mM of $MgCl_2$, 0.2 μM of each primer, 150 μM of each dNTP and 1.5 unit of *Taq* polymerase (Takara, Japan). A 5-min denaturation at 94°C was followed by 35 cycles (30 s at 94°C, 30 s at 57°C and 30 s at 72°C) and a final extension of 10 min at 72°C. The *Hpy*8I restriction enzyme (New England Biolabs, UK) was used for the digestion of the PCR products, which were digested for 3 h at 37°C, with a total volume of 15 μl, containing 5 μl of PCR product, 10 units of *Hpy*8I (MBI, USA) and 1.5 μl of 10× buffer. Digested DNA was separated in an 8% polyacrylamide gel and stained with ethidium bromide.

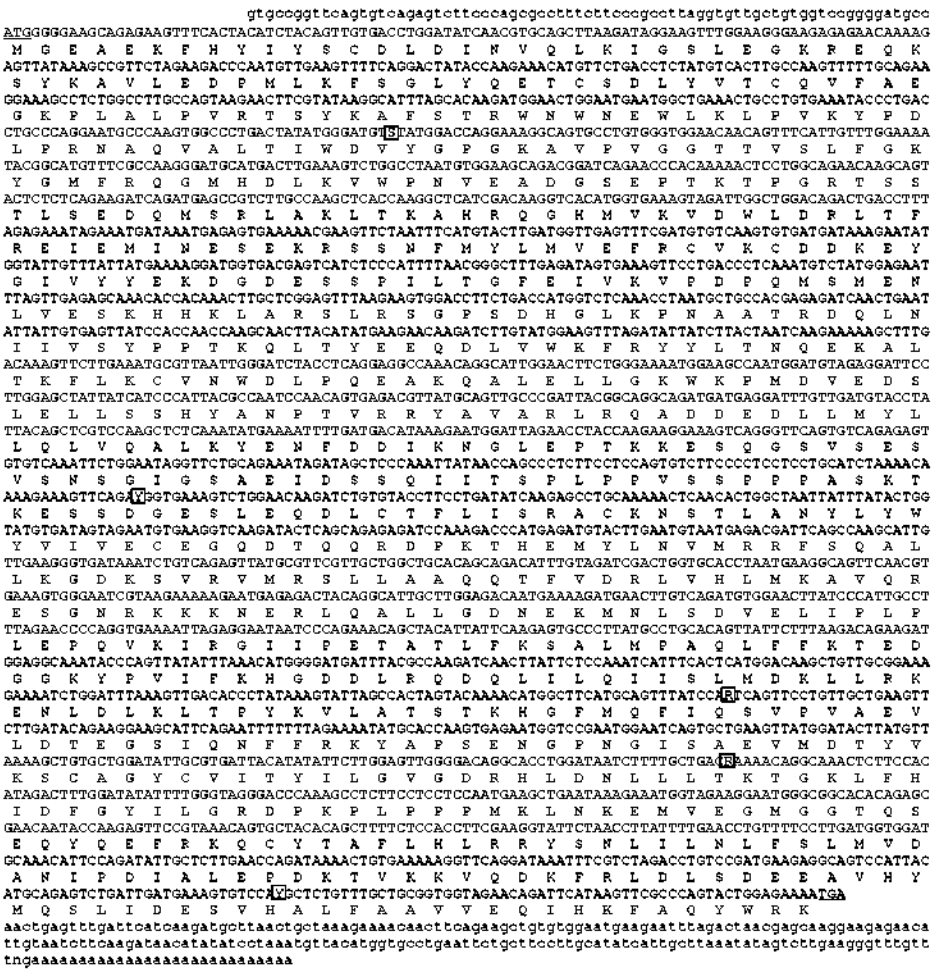

Figure 1. Nucleotide sequence of the full-length mRNA of porcine *PIK3C3* containing 5' and 3' UTRs. The boxed letters indicate mutation points, and the underlined letters are the start and stop codons. Nucleotide numbers located at the right margin indicate the length from the start codon.

Statistical analyses for association with quantitative traits

Variance components models of association and permutation for the exact p-values were applied. Variance components approaches allowed for simultaneous modeling of the means and variances. so that all the information in a set of related individuals could be used to construct an association test.

The following models were evaluated to estimate the total evidence for association:

Null model
Means = μ+s
Variances = V_e+V_g+V_a

Full model
Means = μ+s+x
Variances = V_e+V_g+V_a

The following models were evaluated to estimate the association effects partitioned into between- and within-family components:

Null model
Means = μ+s+b
Variances = V_e+V_g+V_a

Full model
Means = μ+s+b+w
Variances = V_e+V_g+V_a

where μ is the overall mean. s is the mean of sex effects. x is the mean of genotype effects. b is the mean of effects for between-family components. w is the mean of effects for within-family components. V_e is the residual environmental variance component. V_g is the polygenic variance component and V_a is the additive genetic variance component. Note that these expectations do not include any dominance variance.

The likelihood of the data for the complete set of

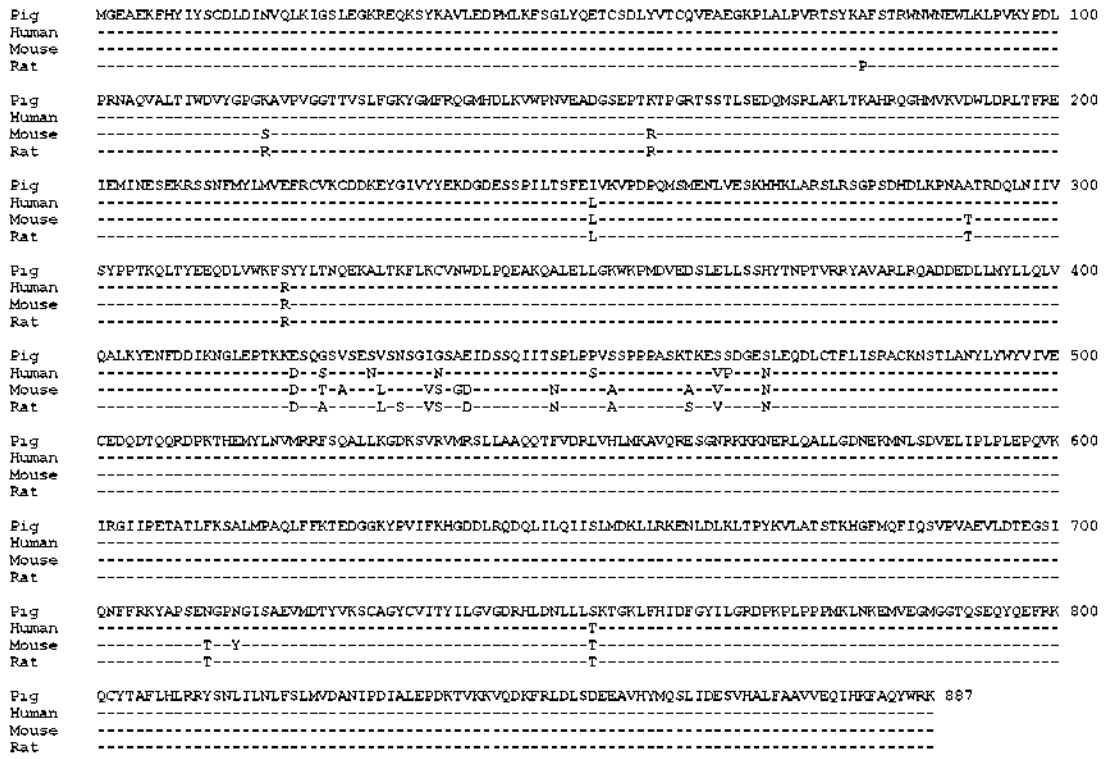

Figure 2. A comparison of the *PIK3C3* amino acid sequences among pigs, humans, mice, and rats. A dash indicates a sequence identity to that of the pig.

parameters and the quantity 2[ln (L_1) - ln (L_0)] (L_0, null-hypothesis likelihood and L_1, alternative-hypothesis likelihood) distributed as χ^2 with df equal to the difference in number of parameters estimated were calculated using the QTDT program (Abecasis et al., 2000).

RESULTS AND DISCUSSION

This study was performed in order to determine and analyze the nucleotide sequence of porcine *PIK3C3* and conduct an association study between genetic polymorphism and quantitative traits, such as growth and fat depot in pigs.

In order to determine a specific part of the mRNA sequence of porcine *PIK3C3*, a primer set (PIK3C3F and R) was first designed from a conserved region between human (GenBank accession no. NM_002647) and mouse (GenBank accession no. NM_181414) mRNA sequences (Table 1); this was done because a pig sequence was not available. Through an alignment of these two mRNA sequences using Blast2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi), conserved positions were selected for primer design. A partial coding sequence (CDS) of *PIK3C3* was obtained using RT-PCR, cloning and DNA sequencing. For obtaining the full-length mRNA sequence, additional primers were designed for 5' and 3' RACE on the basis of the partial CDS that we had previously obtained (Table 1). The full length mRNA and CDS of porcine *PIK3C3* were 2,966 bps and 2,664 bps, respectively, and the gene was presumed to be composed of 25 exons when compared with the human genomic DNA sequence (NC_000018) (Figure 1). Nucleotide sequence identity was shown as 92% between pig and human CDS. After translating the porcine CDS, a comparison of amino acid sequences from four mammalian species were performed (Figure 2). The PIK3C3 of pigs, humans, mice, and rats identically consist of 887 amino acids with sequence identities between the pig and the latter three shown as 99% (human) and 98% (mouse and rat), respectively. In particular, variations between the 421st and 470th amino acids were found with higher frequencies of 60-72.7% than those found in other regions (Figure 2). Genetic distance was calculated using the point accepted mutation model of amino acid mutation suggested by Dayhoff et al. (1978), and a neighbor-joining tree was constructed (Figure 3). The NJ tree showed that the pig formed a clad with humans, indicating that pigs are more closely related to human than with rodents.

Five SNPs-- C339G, C1401T, A2058G, A2256G, and C2604T-- were found from the comparison of mRNA sequences between KNP and Large White pigs. As these SNPs were located at the third position of genetic codes for amino acids, therefore they were deemed silent mutations. Deletion or insertion mutations in the nucleotide sequences

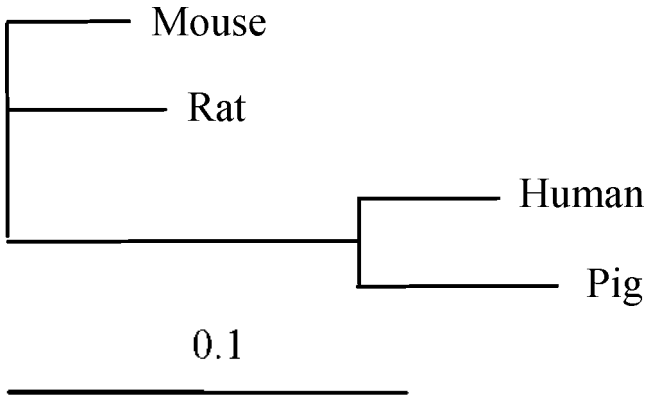

Figure 3. A neighbor-joining tree constructed using the amino acid sequences of four mammalian species. The tree was generated by using the point accepted mutation model of amino acid mutation advocated by Dayhoff et al. (1978).

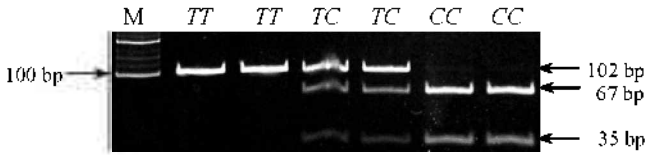

Figure 4. Three genotypes generated from PCR-RFLP analysis with the *Hpy*8I restriction enzyme. M, 100 bp size standard; *TT*, *CT*, and *TT*, three genotypes from the PCR-RFLP analysis.

Table 2. Distributions and allele frequencies of SNP C2604T in four pig breeds

Breeds	Genotypes [1]				Frequencies of *C* allele
	Total	*CC*	*CT*	*TT*	
KNP	20	3	1	16	0.175
Landrace	20	8	8	4	0.6
Duroc	20	10	9	1	0.725
Large White	20	7	3	10	0.425

[1] Genotypes were determined by *Hpy*8I-RFLP analysis.

were not identified. Among the identified SNPs, C2604T was selected for genotyping animals of the four pig breeds. We designed a primer set and performed PCR-RFLP analysis with *Hpy*8I restriction enzyme (Figure 4, Table 2). The frequency of the *C* allele in KNP was shown to be relatively lower, at 17.5%, than those of other breeds. In addition, the highest frequency of *C* allele was observed in the Duroc, which recorded a 72.5% frequency.

One hundred and forty-five F_2 animals produced from a cross between the KNP and Landrace were genotyped for association tests between growth and fat depot traits. In the total association analysis, which doesn't consider transmission disequilibrium, the SNP showed a significant effect ($p<0.05$) on body weight at 30 weeks of age (BW30) and CF, and a highly significant effect ($p<0.01$) on BF (Table 3). Differences in the means between *C* and *T* alleles in BW30, CF and BF were estimated at 7.85 kg, 3.0 kg and 5.3 mm, respectively, indicating that a higher growth rate induces higher BF and CF in the pedigree. The KNP has general characteristics of slow growth and high BF, while the Landrace demonstrates the opposite characteristics. From the results of the association test, it could be inferred simply that the undesirable characteristics, including fast growth but high BF, were generated by the crossing of the two breeds. However, since the animals used for the test were prepared for QTL mapping and extent linkage disequilibrium could exit such populations, the results of such a simple association test might not be completely convincing.

Responding to this, we performed an additional sib-pair analysis using within- and between- family components. For this test, identity-by-descendent probabilities were calculated using an algorithm from the Merlin software (Abecasis et al., 2002), which allows for the inclusion of ungenotyped parents. In this test, the *C* allele still showed positive and significant effects ($p<0.05$) on the BF and CF (Table 4). The effects of the *C* allele on the means of within-family components for CF and BF were estimated by 2.76 kg and 5.07 mm, respectively. As well, the effects of the *C* allele on BW30 revealed in the total association test were not detected in the sib-pair test.

Table 3. Evaluation of the total evidence for association between SNP C2604T and quantitative traits

Traits[1]	df (0) [2]	LnLk (0) [3]	df (x) [4]	LnLk (x)[5]	χ^2 [6]	Significance [7] /p value
BW0	141	-187.87	140	-188.01	0.29	ns/0.60
BW3	134	62.30	133	62.30	0.01	ns/1.00
BW5	133	119.63	132	119.04	1.18	ns/0.70
BW12	140	312.30	139	309.65	5.29	ns /0.06
BW30	141	456.63	140	454.62	4.03	* /0.04
CF	141	302.32	140	300.11	4.42	* /0.01
BF	141	366.92	140	363.93	5.97	* */0.005
IMF	141	121.30	140	120.63	1.35	ns/0.20

[1] BW0, BW3, BW5, BW12 and BW30 are body weight at birth, 3, 5, 12 and 30 weeks of age, respectively. BF, CF and IMF represent backfat thickness, carcass fat and intramuscular fat content, respectively.
[2] The degree of freedom in the null model. [3] The log likelihood estimated by the null model.
[4] The degree of freedom in the full model. [5] The log likelihood estimated by the full model.
[6] Chi square value calculated by 2[LnLk (x)-LnLk (0)].
[7] *, ** indicate differences at the 5% and 1% significance thresholds, respectively and ns means non significance.

Table 4. Evaluation of evidence for association effects using between- and within-family components

Traits[1]	df (0) [2]	LnLk (0) [3]	df (T) [4]	LnLk (T) [5]	χ^{2} [6]	Significance [7] /p value
BW0	139	-188.34	138	-188.38	0.09	ns/0.80
BW3	132	61.39	131	61.31	0.16	ns/0.80
BW5	131	118.87	130	118.65	0.44	ns/0.80
BW12	138	309.44	137	308.39	2.11	ns/0.10
BW30	139	454.78	138	453.85	1.85	ns/0.20
CF	139	292.56	138	290.47	4.17	* /0.04
BF	139	359.11	138	356.41	5.40	* /0.02
IMF	139	118.28	138	118.13	0.29	ns/0.60

[1] BW0, BW3, BW5, BW12 and BW30 are body weight at birth, 3, 5, 12 and 30 weeks of age, respectively. BF, CF and IMF represent backfat thickness, carcass fat and intramuscular fat content, respectively.
[2] The degree of freedom in the null model. [3] The log likelihood estimated by the null model.
[4] The degree of freedom in the full model. [5] The log likelihood estimated by the full model.
[6] Chi square value calculated by 2[LnLk (T)-LnLk (0)].
[7] * Indicates differences at the 5% significance and ns means non significance.

Recent QTL approaches have suggested the existence of BF-related QTLs on SSC6 (Bidanel et al., 2001; Ovilo et al., 2002; Varona et al., 2002; Sato et al., 2003). In particular, Ovilo et al. (2002) reported a significant QTL between SWR1130 and Sw1069 on SSC6q and containing S0228, which was identified as the closest maker to *PIK3C3* in the previous analysis (Kim et al., 2005). The location, as well as the physiological role of *PIK3C3*, reveals the possibility that this gene could be a candidate trait gene for BF-related QTL. For IMF, the *C* allele showed a negative effect, but an insignificant one. This result indicates that if this were a trait gene for the BF-related QTL, IMF would be independent of BF. However, there remains an on-going argument over the existence of a genetic correlation between BF and IMF.

The SNP of porcine *PIK3C3* that was discovered in this study could be utilized as a possible genetic marker for the selection of pigs that possess a low BF and CF at the slaughter weight. However, QTL tests including the SNP, should be demanded to confirm the desired effect. Moreover, since the SNPs found in this study do not fully explain functional changes of the gene because they are silent mutations, additional sequence analysis as well as mutation detection of the promoter region and the CDS should be performed.

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