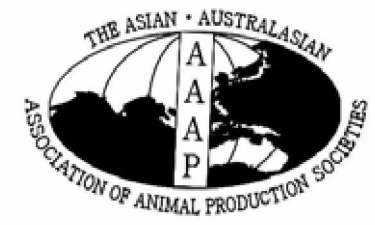

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Molecular Characterization and Chromosomal Mapping of the Porcine AMP-activated Protein Kinase α2 (*PRKAA2*) Gene

Hae-Young Lee[a], Bong-Hwan Choi[a], Jung-Sim Lee, Gul-Won Jang, Kyung-Tai Lee
Ho-Young Chung, Jin-Tea Jeon[1], Byung-Wook Cho[2], Jun-Heon Lee[3] and Tae-Hun Kim*
Division of Animal Genomics and Bioinformatics, National Livestock Research Institute, RDA, Suwon 441-706, Korea

ABSTRACT : AMP-activated protein kinase alpha 2 (*PRKAA2*) plays a key role in regulation of fatty acid and cholesterol metabolism. This study investigated the porcine *PRKAA2* gene as a positional candidate for intramuscular fat and backfat thickness traits in pig chromosome 6. A partial fragment of the porcine *PRKAA2* gene, amplified by PCR, contained a putative intron 3 including a part of exon 3 and 4, comparable with that of human *PRKAA2* gene. Within the fragment, several single nucleotide polymorphisms were identified using multiple sequence alignments. Of these, *Taq*I restriction enzyme polymorphism was used for genotyping various pig breeds including Korean reference family. Using linkage and physical mapping, the porcine *PRKAA2* gene was mapped in the region between microsatellite markers *SW1881* and *SW1680* on chromosome 6. Allele frequencies were quite different among pig breeds. The full length cDNA of the porcine *PRKAA2* (2,145 bp) obtained by RACE containing 1,656 bp open reading frame of deduced 552 amino acids, had sequence identities with *PRKAA2* of human (98.2%), rat (97.8%), and mouse (97.5%). These results suggested that the porcine *PRKAA2* is a positional candidate gene for fat deposition trait at near telomeric region of the long arm of SSC 6. (**Key Words :** *PRKAA2*, Mapping, Candidate Gene, Pig)

INTRODUCTION

Quantitative trait loci (QTL) mapping and candidate gene approaches have been used to find the genes responsible for genetic variation in the traits of interest in farm animals (Rothschild, 1998). Several QTL mapping have been performed for production and meat quality traits using reference family produced by crosses between phenotypically divergent breeds (Andersson et al., 1994; Knott et al., 1998; Rohrer and Keele, 1998; Paszek et al., 1999; Bidanel et al., 2001; Malek et al., 2001). As a result, several research groups identified the same QTL affecting growth and fat deposition traits found in the long arm of SSC6 (de Koning et al., 1999; 2000; Gerbens et al., 2000; Ovilo et al., 2000; Grindflek et al., 2001; Malek et al., 2001).

Using candidate gene analysis, several major genes such as melanocortin 4 receptor (MC4R) (Kim et al., 2000) for growth, ryanodine receptor gene (RYR1) (Fujii, 1991), and heart fatty acid binding protein (hFABP) (Gerbens et al., 1999) for meat quality have been identified for their association with economic traits. Recently, Van Laere et al. (2003) revealed that single nucleotide polymorphism within intron 3 of insulin-like growth factor 2 (IGF2) controls a paternally expressed QTL previously mapped on SSC 2. However, until recently, only a small number of the major genes controlling economically important traits have been identified. Candidate gene analysis also is supplemented by comparative gene analysis that allows researchers to find positional candidate genes in the regions associated with QTL (Rothschild, 1999).

The AMPK (AMP-activated protein kinase) gene family has been assumed to act as a metabolic mediator for glucose and lipid metabolism. The AMPK-activated protein kinase alpha 2 (*PRKAA2*) specially plays a major role as fuel sensor by modulating the activity of the autonomous nervous system (Viollet et al., 2003). Minokoshi et al. (2002) demonstrated that leptin stimulates fatty acid

* Corresponding Author: T. H. Kim. Tel: +82-31-290-1603, Fax: +82-31-290-1602, E-mail: kth6160@rda.go.kr
[1] Gyeongsang National University, Jinju 660-701, Korea.
[2] Department of Animal Science, Busan National University, Miryang, Korea.
[3] Research Center for Transgenic Cloned Pigs, Division of Animal science and resources, Chungnam National University, Daejeon 305-764, Korea.
[a] These authors contributed equally to this work.
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oxidation in muscle through inhibition of ACC (acetyl-CoA carboxylase) by activation of *PRKAA2*.

The AMPK has been known to exist as heterotrimeric complexes comprising a catalytic subunit (α) and two regulatory subunits (β and γ). In mammals, each subunit is encoded by two or three genes (α1, α2, β1, β2, γ1, γ2 and γ3), and at least 12 heterotrimeric combinations are reported (Hardie and Hawley, 2001). Human AMPK gene encodes 552 amino acids and is highly conserved with rat AMPK with identities of 97.3 and 90% at the amino acid and nucleotide levels, respectively (Aguan et al., 1994).

The *PRKAA2* gene was mapped in human chromosome 1p31 (Beri et al., 1994), which is equivalent to the long arm of pig chromosome 6, according to comparative map information between human and pig (Goureau et al., 1996; http://www.toulouse.inra.fr/lgc/pig/compare/SSCHTML/SSC6S.HTM). Therefore, based on its position and biological role, the *PRKAA2* gene can be considered a potential positional candidate gene for QTL affecting fat metabolism and growth traits.

As a first step towards evaluation of the porcine *PRKAA2* gene as a positional candidate controlling QTL for growth and fat deposition traits on SSC 6 in pigs, we report here the molecular cloning, characterization and chromosomal localization of the porcine *PRKAA2* gene.

MATERIALS AND METHODS

Animals

Three-generation reference family was developed from crosses between Korean native pig (five boars) and Landrace (nine sows). The F_1 animals, with 10 sires and 36 dams, were used to produce approximately 550 F_2 animals. All animals were raised under same feeding condition at National Livestock Research Institute (NLRI) in Korea.

To examine allele frequency of polymorphisms, 204 pigs of nine different pig breeds (Berkshire, Duroc, Korean native pig, Korean wild boar, Landrace, Min pig, Wuzhishan pig, Xiang pig, and Yorkshire) were investigated in the study.

Amplification of porcine *PRKAA2* gene fragment and mutation detection

Primers for amplification of a fragment of pig *PRKAA2* gene were designed from a published partial sequence of the pig *PRKAA2* gene (GenBank accession no. U12148). The primer sequences were selected, as follows: forward primer: 5'-TGG TAA TGG AAT ATG TGT CTG G-3'; reverse primer: 5'-ATC CAC GGC AGA GAG AAT CT -3'.

Polymerase chain reaction (PCR) was performed in 25-μl reactions with 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* each dNTP, 10 pmol each primer, 1.0 units Taq DNA polymerase (TaKaRa, Japan), and 50 ng genomic DNA. Thermal cycling conditions included an initial denaturation for 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 62°C and 1 min at 72°C, and a final extension of 10 min at 72°C in GeneAmp PCR System 9600 (Applied Biosystems, USA).

For detection of nucleotide differences, direct sequencing of the PCR products was performed using Big Dye Terminator Cycle Sequencing Ready Reaction kit Ver 3.0 (Applied Biosystems, USA) and the ABI 377 DNA sequencer (Applied Biosystems, USA). The sequences were compared to find single nucleotide polymorphisms from five different breeds, namely: Korean native pig, Berkshire, Duroc, Landrace, and Large White.

Physical and genetic linkage mapping

Using the primer set to amplify a porcine *PRKAA2* gene fragment, chromosomal localization of the *PRKAA2* gene was conducted by PCR analysis of a porcine×rodent somatic cell hybrid panel (Yerle et al., 1996) as well as a porcine whole genome radiation hybrid panel (Yerle et al., 1998). PCR results were analyzed using the interpreting web pages of the National Institute for Agricultural Research (INRA) (http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm, and http://imprh.toulouse.inra.fr/).

Furthermore, two-point and multipoint linkage analyses were performed using the genotypes of the three-generation Korean reference family and the CRI-MAP software version 2.4 (Green et al., 1990).

Cloning of full length cDNA by rapid amplification of cDNA ends (RACE)

The porcine mRNA for RACE experiment was extracted from the porcine *longissimus dorsi* muscle using FastTrack™ 2.0 kit (Invitogen Co., CA, USA). To amplify the 3' and 5' cDNA ends of the *PRKAA2* gene, SMART™ RACE cDNA Amplification kit (CLONTECH Laboratories Inc., CA, USA) was used according to the manufacturer's instruction.

The porcine *PRKAA2* specific internal primer sequences for 5'RACE were used, as follows: 5'- 5'-CAT CAA CTG ACA GGC CAT AAA GTG GCA-3' for the first round and 5'- ATA CCA GGT GAT CAG CAC TCC GAC AGA -3' for the second round.

For 3' RACE, internal primers used for the first and the second round amplifications were 5'- CCA TAT GCC TGT GAC AGT AAT CCA CGG -3' and 5'- GCC TGG CTT CCA TCT CTT CAA CCC G -3', respectively.

RESULTS

The amplification of porcine *PRKAA2* gene fragment and mutation detection

The primers for amplification of a fragment from a

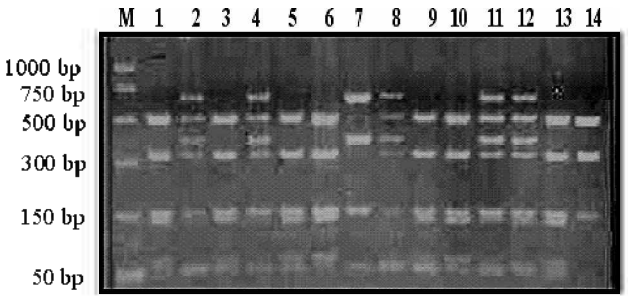

Figure 1. PCR-RFLP patterns for the porcine *PRKAA2* gene digested with *Taq*I restriction enzyme. M: standard size marker; AA genotype - lanes 1, 3, 5, 6, 9, 10, 13, 14; Aa genotype - lanes 2, 4, 8, 11, 12; aa genotype - lane 7.

porcine *PRKAA2* gene were designed based on the published sequence at GenBank (GenBank accession no. U12148). Approximately 1,200 bp of the porcine *PRKAA2* gene fragment was amplified by PCR and sequenced. The sequence of the PCR product was verified by comparison with *PRKAA2* gene sequences of the pig (GenBank accession no. U12148), and the human (GenBank accession no. NM006252). The fragment contained a putative intron 3 including a part of exon 3 and 4 according to the comparison with that of human *PRKAA2* gene (GenBank accession no. AY877365).

Several single nucleotide polymorphisms within the PCR fragment were found using multiple sequence alignments, including a *Taq*I restriction enzyme recognition site. There were two nucleotide substitution sites that can be recognized by *Taq*I restriction endonuclease; these were 506th and 969th bp of the sequence. Thus, the "A" allele has 505, 324, 156, 138, and 61 bp bands, and the "a" allele has 643, 384, and 156 bp bands (Figure 1).

Physical mapping and genetic linkage

Physical map location of *PRKAA2* gene was assigned to SSC6 using somatic cell hybrid (SCH) and INRA-University of Minnesota porcine Radiation Hybrid (ImpRH) panels (Yerle et al., 1996; Yerle et al., 1998).

Initial screening with SCH panel showed that the hybrid clones 9, 10, 18, and 26 were positive harboring the region of chromosome 6, as follows: 6(1/2)q31 (correlation = 0.85), 6(1/2)q32 (correlation = 0.88), 6q33-q34 (correlation = 0.88), and 6(1/2)q35 (correlation = 0.88), respectively. The *PRKAA2* gene was mapped clearly in these regions based on the mapping statistics (error risk, <0.5%, maximal correlation, 0.87).

The porcine *PRKAA2* gene was further assigned with 118 clones of IMpRH panel to find out the more precise map location. Multipoint analysis revealed that significantly linked markers adjacent to the *PRKAA2* gene were SW322, SW1069, and SW1680 on SSC6 with LOD scores of 12.25, 8.49, and 8.48, respectively (Figure 2) (Hawken et al., 1999).

Linkage mapping by two- and multipoint analysis showed that the most significant linkages between *PRKAA2* and markers were obtained from SW1881 (recombination fraction = 0.20; LOD = 7.65) and SW1680 (recombination fraction = 0.08; LOD = 15.39) on chromosome 6 (Figure 2).

Genotype frequency of various pig breeds and Korean reference family

The frequency of *Taq*I-RFLP genotype was determined in 204 unrelated animals from nine breeds, namely: three Chinese breeds (Min pig, Wuzhishan pig, and Xiang pig).

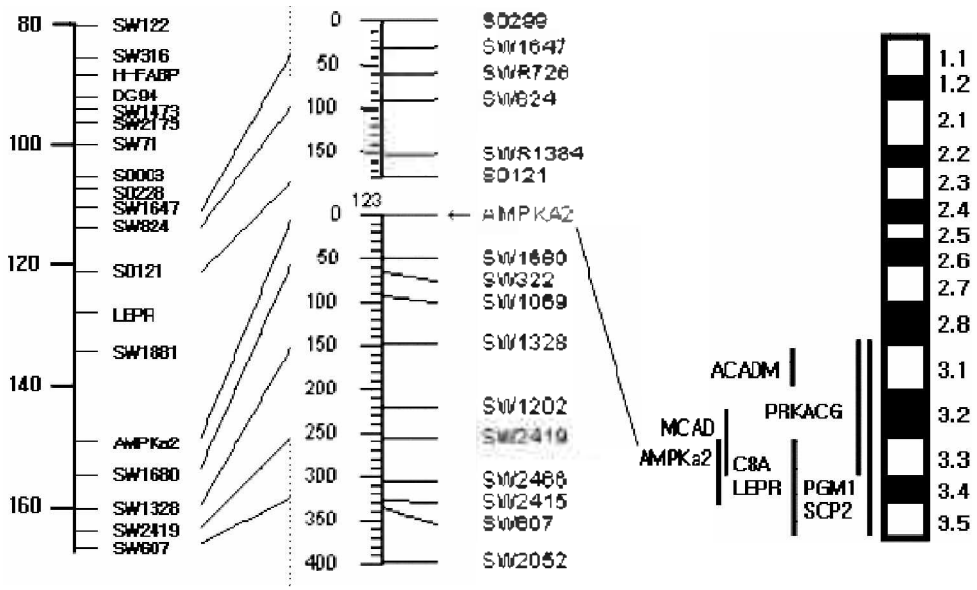

Figure 2. Physical and genetic maps corresponding to a long arm of SSC 6 including the porcine *PRKAA2* gene.

Table 1. Allele frequencies of a *Taq*I PCR-RFLP genotypes in different pig breeds

Breeds	n	Frequencies							
		Genotype						Gene	
		AA		Aa		aa		A	a
		n	%	n	%	n	%		
Berkshire	32	29	90.6	2	6.3	1	3.1	0.94	0.06
Duroc	64	62	96.9	2	3.1	0	0.0	0.97	0.03
Korean native pig	30	23	76.7	6	20.0	1	3.3	0.87	0.13
Korean wild boar	6	0	0.0	0	0.0	6	100.0	0.00	1.00
Landrace	38	27	71.1	10	26.3	1	2.6	0.84	0.16
Min pig	8	4	50.0	3	38.0	1	12.0	0.69	0.31
Wuzhishan	18	0	0.0	1	5.6	17	94.4	0.02	0.98
Xiang pig	27	0	0.0	2	7.4	25	92.6	0.04	0.96
Yorkshire	99	56	56.6	39	39.4	4	4.0	0.76	0.24
German pietrain	20	5	25.0	13	65.0	2	10.0	0.58	0.42
Hampshire	29	28	96.6	1	3.4	0	0.0	0.98	0.02

Table 2. Interspecies identities of the *PRKAA2* coding sequence and the predicted amino acid sequence

Species	Identity with the porcine *PRKAA2* gene (%)	
	DNA	Amino acid
Human[a]	94	98.2
Rat[b]	89	97.8
Mouse[c]	89	97.5

[a] Beri et al., 1994; [b] Gao et al., 1995; [c] GenBank accession number XM_131633.

two Korean breeds (Korean native pig and Korean wild boar), and four Western breeds (Berkshire, Duroc, Landrace, and Yorkshire).

As shown in Table 1, the Asian breeds had higher frequency of "a" allele compared with that of Western breeds. The Korean wild boar, Wuzhishan pig, and Xiang pig had 1.00, 0.98 and 0.96, respectively. The "a" allele frequency of Korean native pig and Min pig were 0.13 and 0.31, respectively. The Western breeds had much lower frequencies, with 0.00 in Duroc; and 0.13 in Landrace and Yorkshire.

For linkage mapping, the frequencies of the *Taq*I PCR-RFLP genotypes for F_2 animals of the Korean reference family were determined. Among F_2 animals analyzed in this study, frequencies of genotype AA, Aa, and aa were 61.6 (n = 337), 31.3 (n = 171), and 7.1% (n = 39), respectively. Among founder animals in the Korean reference family, eight pigs (four boars and four sows) were found to have genotype AA; five pigs (one boar and four sows) had genotype Aa; and one sow had genotype aa.

Cloning and sequence analysis of porcine *PRKAA2* gene

Full-length cDNA of the porcine *PRKAA2* gene was identified using 5' and 3' RACE. The complete sequence of 2145 bp was deposited at GenBank (Accession no. AY159788). The coding sequence of the porcine *PRKAA2* cDNA was predicted using ORF finder program in NCBI (http://www.ncbi.nlm.nih.gov/). The porcine *PRKAA2* gene had a total of 552 amino acids.

The porcine *PRKAA2* amino acid sequence was almost identical with that of the *PRKAA2* gene of human (98.2%), rat (97.8%), and mouse (97.5%). The coding sequence of the porcine *PRKAA2* gene also had some identity with that of human (94%), rat (89%), and mouse (89%) (Table 1 and Figure 2).

The *PRKAA2* gene was highly conserved among the species except for variations of c-terminal. By comparing with human *PRKAA2* genomic sequences, the porcine *PRKAA2* gene was predicted to have nine exons (http://www.ncbi.nlm.nih.gov/genome/seq/page.cgi?F=HsBlast.html&&ORG =Hs).

DISCUSSION

The AMPK plays a major role in the regulation of fatty acid and cholesterol metabolism. Using linkage analysis, the porcine *PRKAA2* gene was mapped in the region between SW1881 and SW1680 on SSC6. The human and bovine *PRKAA2* genes were mapped in chromosome 1p31 (Beri et al., 1994; Aguan et al., 1994) and chromosome 3 (Mckay et al., 2003), respectively.

Several QTL affecting growth and fat deposition were identified near the *PRKAA2* locus of SSC6 (Grindflek et al., 2001; de Koning et al., 1999; 2000; Gerbens et al., 2000; Ovilo et al., 2000; Malek et al., 2001). A significant QTL affecting BFT, IMF content and eye muscle area was detected in the intervals between S0228 and SW1881 on SSC6 in an F_2 cross between Iberian and Landrace pigs (Ovilo et al., 2000).

Suggestive QTL for tenth rib back fat was mapped in the region between SW322 and SW2052 (Malek et al., 2001); for fatness, in the intervals between S0121 and SW322 (Bidanel et al., 2001); and for IMF content and BFT, in the intervals between S0003 and SW2419 (Gerbens et al., 2000) on SSC 6. It is assumed that those QTL were located closely in the same region where the *PRKAA2* was located.

Grindflek et al. (2001) reported that a significant QTL

```
Pig     :  1  MAEKQKHDGRVKIGHYVLGDTLGVGTFGKVKIGEHQLTGHKVAVKILNRQKIRSLDVVGK 60
Human   :  1  ------------------------------------------------------------ 60
Rat     :  1  ------------------------------------------------------------ 60
Mouse   :  1  ------------------------------------------------------------ 60

Pig     : 61  IKREIQNLKLFRHPHIIKLYQVISTPTDFFMVMEYVSGGELFDYICKHGRVEEMEARRLF 120
Human   : 61  ------------------------------------------------------------ 120
Rat     : 61  -------------------------------------------------------V------ 120
Mouse   : 61  -------------------------------------------------------V------ 120

Pig     : 121 QQILSAVDYCHRHMVVHRDLKPENVLLDAQMNAKIADFGLSNMMSDGEFLRTSCGSPNYA 180
Human   : 121 ------------------------------H------------------------------ 180
Rat     : 121 ------------------------------------------------------------ 180
Mouse   : 121 ------------------------------------------------------------ 180

Pig     : 181 APEVISGRLYAGPEVDIWSCGVILYALLCGTLPFDDEHVPTLFKKIRGGVFYIPEYLNRS 240
Human   : 181 ------------------------------------------------------------ 240
Rat     : 181 ------------------------------------------------------------ 240
Mouse   : 181 -------------------------------------------------------D----- 240

Pig     : 241 VATLLMHMLQVDPLKRATIKDIREHEWFKQDLPSYLFPEDPSYDANVIDDEAVKEVCEKF 300
Human   : 241 ------------------------------------------------------------ 300
Rat     : 241 I----------------------------------------------------------- 300
Mouse   : 241 I----------------------------------------------------------- 300

Pig     : 301 ECTESEVMNSLYSGDPQDQLAVAYHLVIDNRRIMNQASEFYLASSPPTGSFMDDSAMHIP 360
Human   : 301 --------------------------I--------------------S------------ 360
Rat     : 301 --------------------------I---------------------------M----- 360
Mouse   : 301 --------------------------I--------------------S------------ 360

Pig     : 361 PGLKPHPERMPPLIADSPKARCPLDALNTTKPKSLAVKKAKWHLGIRSQSKPYDIMAEVY 420
Human   : 361 ------------------------------------------------------------ 420
Rat     : 361 ------------------------------------------------------------ 420
Mouse   : 361 ------------------------------------------------------AC------- 420

Pig     : 421 RAMKQLDFEWKVVNAYHLRVRRKNPVTGNYVKMSLQLYLVDNRSYLLDFKSIDDEVLEQR 480
Human   : 421 ------------------------------------------------------------V--- 480
Rat     : 421 ------------------------------------------------------------V--- 480
Mouse   : 421 ------G-----------------------------------S--------------V--- 480

Pig     : 481 SGSSTPQRSCSAAGLHRPRSSLDSVTAESHSLSGSLSGSLTGSMLPSVPPRLGSHTMDFF 540
Human   : 481 ----------------------F--T-----------T------T-S--S----------- 540
Rat     : 481 ----------------------V--S---N-------T------T----S----------- 540
Mouse   : 481 -----------------A---F--S---N------------------------------- 540

Pig     : 541 EMCASLITTLAR 552
Human   : 541 ------------ 552
Rat     : 541 --------A--- 552
Mouse   : 541 --------A--- 552
```

Figure 3. Amino acid sequence alignment of the AMP-activated protein kinase alpha2 of pig, human, rat and mouse. Human (Genbank accession no. MN006252; Beri et al., 1994), rat (accession no. Q09137; Gao et al., 1995), and mouse (accession no. XM131633; unpublished data). Dash (-) indicates the homology of the amino acid sequences.

for IMF content was found in the region between SW1823 and S0003 on SSC6 in a commercial pigs. Interestingly, the paternally expressed QTL affecting IMF content on SSC6 had been detected in the region between

SW316 and S0003 with a high significance level (De Koning et al., 2000). This region was a suggestive evidence of Mendelian QTL for IMF content, without the imprinting effect in the model (De Koning et al., 1999). Those QTL also are likely to be closely linked with the *PRKAA2* locus identified in this study.

Furthermore, leptin, a hormone secreted by adipocytes, plays a major role in the regulation of food intake and energy expenditure (Friedman and Halaas, 1998). Leptin stimulates fatty acid oxidation and glucose uptake in skeletal muscles (Muoio et al., 1997; Minokoshi et al., 2002).

On the other hand, AMPK stimulates the oxidation of fatty acids in skeletal muscle by inhibiting the activity of

acetyl coenzyme A carboxylase (ACC). Thus, AMPK plays as a principal mediator of effects of leptin on fatty acids metabolism in muscle (Hardie et al., 1998; Minokoshi et al., 2002). AMPK is highly conserved in mammals, yeast (SNF1) and plants (RKIN1) suggesting its significant biological role (Carling et al., 1994).

PRKAA2 gene was mapped in more distal region away from the leptin receptor (LEPR) approximately 21.7 cM; and from the human heart-type cytoplasmic fatty acid-binding protein (H-FABP), 61.9 cM.

H-FABP and LEPR are considered as candidate genes associated with growth and fatness in the long arm of SSC 6. The polymorphism in H-FABP gene was associated with IMF as well as BFT in Duroc (Gerbens et al., 1999), and it was mapped in the region between markers SW316 and S0003 on SSC6 (Gerbens et al., 2000).

Although there has been no reported polymorphism in LEPR associated with other traits in pig, LEPR also is considered as one of candidate genes associated with growth and fat trait because of its position and biological function.

In addition, several other genes including ACADM, PRKACG, PGM1, SCP2, C8A, and MCAD were mapped in this region (http://www.toulouse.inra.fr/lgc/pig/cyto/genmar/htm/6GM. HTM).

Other candidate genes on other chromosomes for growth and fatness, found to be associated with economically important traits in pigs, include MC4R (Kim et al., 2000); RYR1 (Fujii et al., 1991); RN locua (Le Roy et al., 2000; Milan et al., 2000); and IGF2 (Van Laere et al., 2003). Van Laere et al. (2003) reported the relationship between single nucleotide polymorphism (SNP) in intron 3 of IGF2 and a QTL effect.

These results are suggested that the porcine *PRKAA2* is a potential positional candidate gene controlling QTL for fat deposition trait near telomeric region of the long arm of SSC 6. However, in order to develop a marker for marker-assisted selection, it needs to discovery and evaluate the single nucleotide polymorphisms within exons or regulatory region in the porcine *PRKAA2*.

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