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A Novel Single Nucleotide Polymorphism of the Leptin Receptor Gene Associated with Backfat Thickness in Duroc Pigs

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Fatness is one of the most important economic traits in pigs. The leptin receptor (*LEPR*) gene may be a potential candidate for the fatness quantitative trait locus (QTL) on porcine chromosome 6, due to its position and physiological role. Thus, this study was carried out to evaluate the associations between structural variants in the *LEPR* gene and economic traits in pigs. We obtained an approximately 114-kb sequence containing the complete genomic DNA of the porcine *LEPR* gene, using shotgun sequencing of a bacterial artificial chromosome clone. We report the complete genomic structure of the porcine *LEPR* gene. Dozens of transcription factor-binding sites were found in the 1.2 kb upstream region from the transcription start point. An association study was performed with 550 Duroc pigs for 24 single-nucleotide polymorphisms (SNPs), including 6 SNPs within exons and 18 SNPs within the putative 5′ regulatory region of the porcine *LEPR* gene. Among them, one SNP (-790C/G) was significantly associated with backfat thickness and lean meat percentage, whereas the others, including two SNPs with missense polymorphisms, had no effect on any phenotype. These results suggest that SNP -790C/G may be a useful marker for genetic improvements of fatness and leanness in Duroc pigs.

Key words : Economic trait, leptin receptor, pig, single nucleotide polymorphism

Introduction

Quantitative trait locus (QTL) mapping has been performed to detect chromosomal regions that are associated with production and meat quality traits by crossing phenotypically divergent breeds. To date, more than 6,800 QTLs representing 585 overlapping phenotypic traits have been deposited in pig QTLdb (http://www.animalgenome.org/cgi-bin/QTLdb/SS/index). Moreover, several QTLs for growth and fat deposition traits have been identified in a similar region of swine chromosome (SSC) 6 [3, 6, 8, 11, 13, 17, 18, 25]. Subsequently, great efforts have been made to find causal mutations controlling the QTLs through fine mapping or positional candidate gene approaches. However, definite conclusions have not yet been drawn based on those results [1, 12, 19, 20, 25]. The leptin receptor (*LEPR*) gene is well known a potential positional candidate gene controlling QTL for growth and fatness traits in the long arm of SSC6 because of its position and biological function.

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Leptin, produced primarily in adipose tissue, is involved in the regulation of feed intake, energy balance, and reproduction in mammals [5]. Leptin signaling is mediated via the LEPR, which belongs to the class I cytokine family [23]. *Leptin* and *LEPR* genetic variants are associated with obese phenotypes in humans and mice, and the two genes are expected to influence fat deposition in pigs [4]. Associations between *LEPR* variants and reproductive [2] and fatness traits [12] have been reported in pigs. Ovilo et al. (2005) found a significant association between *LEPR* alleles and backfat thickness in a narrow region (130 - 132 cM) of chromosome 6 [20]. In recent, Uemoto et al. (2012) detected a significant SNP (c.2002C>T) in exon 14 on fatness traits [24]. All association studies on *LEPR* have been performed between exonic or intronic mutations and phenotypes in pigs. A few cDNA sequences and partial sequences of the porcine *LEPR* have been deposited in GenBank (e.g., AF092422), but the complete genomic organization has not been characterized. Moreover, the 5′ regulatory region of the porcine *LEPR* gene sequence has not been published.

Therefore, this study was carried out to evaluate the por-

cine *LEPR* gene as a positional candidate controlling the QTL for growth and fat deposition traits on SSC6. In addition, we report the complete genomic structure containing the 5′ regulatory region of the porcine *LEPR* gene.

Materials and Methods

Ethics statement

The study protocol and standard operating procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science (No. 2009-077, C-grade).

Isolation of a bacterial artificial chromosome clone containing the porcine *LEPR* gene

A bacterial artificial chromosome (BAC) clone containing the *LEPR* gene was obtained from the Korean native pig (KNP) BAC library [10] using a polymerase chain reaction (PCR) screening method. A BAC clone containing the *LEPR* gene was screened with LEPR-CA STS (UniSTS: 253565, Forward: 5′-TTCCAGAAACATAAGACACGCG-3′, Reverse: 5′- GACCAATTCTAAATTTCAACCAGAGG-3′). A shotgun library of the screened BAC clone, KNP_645H8, was constructed using the pUC19 plasmid vector (Qbiogene, Irvine, CA, USA). The sequence was obtained using an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM3730 Genetic Analyzer (AppliedBiosystems). The DNA sequences were assembled with Phred and Phrap software (University of Washington). The assembled sequence was deposited into GenBank of NCBI (FN673752).

Structural analysis of the porcine *LEPR* gene

Exon-intron boundaries of the *LEPR* gene on the sequence of BAC clone were determined by comparing with the porcine mRNA sequence (AF092422). Potential transcription factor-binding sites in the 5′ upstream region were predicted using the TRANSFAC 8.4 professional program. The putative promoter sequence of the porcine *LEPR* gene was aligned with the human (AC097063) and mouse (AL929373) sequences using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to investigate consensus sequences within the promoter regions among species.

Single nucleotide polymorphism discovery

Single nucleotide polymorphisms (SNPs) within exons and a putative promoter region were detected by direct sequencing of the samples pooled from five different breeds, including the Korean native pig, Berkshire, Duroc, Landrace, and Yorkshire. Eleven pairs of primers covering 1.2 kb upstream and 11 exon regions were designed based on the BAC clone sequence obtained (Table 1). The PCR reaction was performed in a 50 µL final volume containing 50 ng template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM $MgCl_2$, 0.2 µM each primer, 100 μM each dNTP, and one unit *Taq* DNA polymerase (GeNet Bio, Korea). Reaction profiles included a 5 min denaturation step at 94°C followed by 35 cycles each consisting of 30 s at 94°C, 30 s at the annealing temperature (Table 1), 1 min at 72°C, and then a final 10 min extension step at 72°C using a PTC-225 Peltier Thermal Cycler (MJ Research, Waltham MA, USA).

The PCR products were cleaned up with a QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and sequenced with the respective PCR primers using BigDye Terminator Cycle Sequencing Kit version 3.2(Applied Biosystems, USA) and an 3730XL DNA Analyzer (AppliedBiosystems). SNPs were identified by multiple alignments of sequence chromatograms generated with each primer pair using SeqMan program of Lasergene package (DNASTAR, USA).

Genotyping and phenotypes

In total 1,014 pigs from nine different breeds including western breeds (Berkshire, Duroc, Landrace, and Large White), the Korean native pig, the Korean wild pig, and Chinese breeds (Xiang, Min, and Wuzhishan pig) were used to investigate the allelic frequencies of SNPs. The traits analyzed in this study were average daily weight gain, feed efficiency, backfat thickness, and lean meat percentage. Blood samples were collected from 550 Duroc pigs at the Pig Breeding Stock Evaluation Center of the Korean Swine Association in Korea for the association test. Genomic DNAs were extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). PCR reactions were performed in a 25 µl final volume containing 25 ng template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM $MgCl_2$, 0.2 µM each primer, 100 µM each dNTP, and one unit *Taq* DNA polymerase (GeNet Bio) for genotyping of the 18 SNPs in the promoter region and the 6 SNPs in the exon region (Table 2). Thermal cycling parameters were defined as follows: pre-denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing temperature for 30 s (Table 1), 72°C for 1 min, and then a final step at 72°C for 10 min

Table 1. List of primer sequences used to amplify the porcine *LEPR* gene

Primer name	Regions	Sequences	Product size (bp)	Annealing temp.
1FR	5UTR/Exon1/intron1	ATGTAAAATAGCATTGCATGA TTAGCCATTTAAAGGAAACAG	350	53
2FR	Exon2/ intron2	TGCATTTGACTTGGCATATCC CAGTCCTATCTGCCAGTCGAT	646	62
3FR	intron3/Exon4/intron4	AAGCTGGGTGTCCAGAAGAG CAGGGTTGGTCAACGTGAGA	428	62
4FR	intron4/Exon5/intron5	ACCAATATTTTGTTTCCGTAA TCTTTTAGCTGCTTCGCAAT	354	55
5FR	Intron6/Exon7/Intro7 /Exon8/intron8	CAGCTCCAGTTCTGCAGTCTCCTAGCG TCACCAATTTAATCCCTCGAA	868	55
6FR	Intron11/Exon12/intron12	TGAAAAATAATCCTTCCAAGA GCAGAGTCCAGAAATGACTT	232	54
7FR	Intron12/Exon13/intron13	GTCCTGGATTCTGCAACGCTT GAAAGAGAGACTTTCCGACAC	466	62
8FR	Intron13/exon14/intron14	GCCTTGTTCAGTGATACAGCAG TACAGTGAGCTTACCGTGGAT	482	59
9FR	Intron15/Exon16/intron16	CCTAGTTGGATTCGTTTCTG AGAATACTTCAGTGTTTCCGAAT	265	54
10FR	intron17/Exon18/3UTR	CCCAGTTGATTTGTGACGACT GGAGGATTTTGATGTCAGCGA	811	65
proLEPR	Upstream region	TCCACCCAGATTAATTTTCA TACCCCAATGTAACAAAGCC	1205	53

Table 2. Exon-intron organization of the porcine *LEPR* gene

Exon	cDNA (AF092422)	Exon size (bp)	Intron	Intron size (kb)	Exon	cDNA (AF092422)	Exon size (bp)	Intron	Intron size (kb)
1	1-55	55	1	5.084	10	1619-1767	149	10	1.114
2	56-385	330	2	1.878	11	1768-1927	160	11	0.615
3	386-509	124	3	7.174	12	1928-2010	83	12	3.355
4	510-718	209	4	4.291	13	2011-2227	217	13	1.956
5	719-8864	146	5	2.573	14	2228-2410	183	14	1.667
6	864-1009	145	6	5.874	15	2411-2506	96	15	1.579
7	1010-1300	291	7	0.166	16	2507-2609	103	16	1.455
8	1301-1418	118	8	2.627	17	2610-2685	76	17	13.974
9	1419-1618	200	9	3.427	18	2686-4032	1347		

using a PTC 225 Peltier Thermal Cycler (MJ Research). Genotypes of 550 samples were determined by PCR-restriction fragment length polymorphism (PCR-RFLP) analyses using *Tsp*509I, *Hpyc*H4III, *Nde*I, *Aci*I, *Dra*III, and *Sau*3AI (Table 2) for exonic mutations and direct sequencing for SNPs within the upstream region.

Statistical analysis

Data were analyzed with the general linear model procedure using SAS (SAS Institute, Cary, NC, USA) to test the effect of each genotype on performance traits. Mean differences were established based on the least squares means comparison. A *p*-value <0.05 was considered significant. The formula for analyzing traits was $Y=X\beta+M+e$, where Y is the phenotype vector; X and β are the design matrix and solution vector for fixed effects including year/season of birth and gender and the performance testing period (days) covariate, respectively; M is a 3×1 vector of the genotype effects; and e is a residuals vector.

Results and Discussion

Positional and biological candidate gene studies may help identify genes responsible for phenotypic variation. In particular, positional candidate gene analyses are intended to evaluate whether a positional candidate gene is effective on some QTL or closely linked to the QTL. Association studies yield significant results when an SNP within a candidate gene is a causal variation or in linkage disequilibrium with it [20]. This study was conducted to evaluate the influence of variations in the porcine *LEPR* gene on production traits in pigs. This is the first report providing evidence for the effect of one SNP in the 5′ regulatory region on production traits in a Duroc population. Moreover, we revealed the complete genomic sequences including the 5′ regulatory region in the porcine *LEPR* gene.

Genomic structure of the porcine *LEPR* gene

We screened a BAC clone (KNP_645H8) containing the LEPR-CA microsatellite marker and obtained an approximate 114 kb sequence (GenBank acc. no. FN673752) using the shotgun sequencing method. The complete genomic structure of the *LEPR* gene including a putative promoter region was revealed by comparison with the porcine cDNA sequence (AF092422). As shown in Table 2, the porcine *LEPR* gene was organized with 18 exons spanning approximately 63 kb of genomic DNA. Exon 18 was the longest, representing 1,347 bp. The intron sizes of the *LEPR* gene were in the range of 166 bp to approximately 14 kb. The translation initiation codon was located in exon 1. In addition, all exon/intron boundary sequences followed the GT-AG rule for splice-donor and acceptor sites reported by Jacob and Gallinaro (1989) [9]. The putative transcriptional factor binding sites included activating protein-1, CCAAT-enhancer-binding protein (C/EBP)-α, peroxisome proliferator-activated receptor (PPAR)-α, retinoid X receptor-α, and nuclear factor (NF)-kB (data not shown). Multiple potential transcription factor-binding sites were also identified in the porcine *LEPR* promoter region and might be responsible for transcriptional regulation of the porcine *LEPR* gene. The TATA box (CTTATATATA) was predicted in the region 52 bp upstream from the transcription initiation start point by the WebGene program.

Discovery of SNPs

A total of eleven primer sets were used to amplify ten exonic regions and the upstream region of exon1 of the porcine *LEPR* gene (Table 1). Thirty six SNPs were identified by direct sequencing. Within 1,345 amino acids of the CDS of the porcine *LEPR* gene (AF092422), two nonsynonymous (I73L and T220A) and four synonymous (L243L, S724S, S775S, and D947D) mutations were found within the exon region (Table 3). Eighteen SNPs were polymorphic in the 5′ regulatory region of the Duroc population. Thirty SNPs were initially discovered in the 5′ regulatory region, of which only 18 loci were polymorphic in the Duroc breed (Table 4). Furthermore, most of the SNP sites were involved in a putative transcription factor-binding sequence (Table 4). The causal mutation in the regulatory region is critical for regulating gene expression, because transcriptional control is mediated mainly through the interactions of regulatory transcription factors with their cognate enhancer elements Novina & Roy, 1996).

Several studies for the effect of *LEPR* gene variants on phenotypes in pigs have been performed [2, 12, 15, 16, 19, 20]. Ovilo et al. (2005) reported that a missense mutation

Table 3. Allelic frequencies of the porcine *LEPR* exonic variations in nine pig breeds ND: Not determined

Breeds	N	Allele frequency (%)											
		A232T (I73L)		A673G (T220A)		A744G (L243L)		C2187T (S724S)		A2340G (S775S)		C2856T (D947D)	
		A	T	A	G	A	G	C	T	A	G	C	T
Korean native pig	24	ND	ND	0.15	0.85	0.85	0.15	0.50	0.50	0.55	0.45	0.45	0.55
Landrace	108	0.10	0.90	0.08	0.92	0.80	0.20	0.78	0.22	0.73	0.27	0.13	0.87
Duroc	550	0.20	0.80	0.00	1.00	0.68	0.32	0.71	0.29	0.66	0.34	0.22	0.78
Berkshire	77	0.09	0.91	0.09	0.91	0.87	0.13	0.69	0.31	0.75	0.25	0.08	0.92
Large White	182	0.02	0.98	0.27	0.73	0.99	0.01	0.43	0.57	0.52	0.48	0.26	0.74
Korean wild pig	19	ND	ND	0.16	0.84	1.00	0.00	0.81	0.18	0.65	0.35	0.36	0.63
Xiang	27	ND	ND	0.44	0.56	1.00	0.00	0.06	0.94	0.25	0.75	0.19	0.81
Min	7	ND	ND	0.33	0.66	1.00	0.00	0.08	0.92	0.45	0.55	0.31	0.69
Wuzhishan	20	ND	ND	0.65	0.35	1.00	0.00	0.15	0.85	0.58	0.42	0.69	0.31

Table 4. SNPs position, allele frequencies and transcription factor binding sites of 5′ regulatory region of the porcine *LEPR* gene in Duroc breed

	Allele (1/2)	Allele frequency		Transcription factors	Score	Sequence
		1	2			
-91	A/C	0.55	0.45	-	-	-
-132	G/A	0.99	0.01	EMF	100	**CATTTG**
-201	G/A	0.99	0.01	-	-	**-**
-203	C/T	0.62	0.38	GATA-1	100	AAT**C**T
-230	A/T	0.99	0.01	T3R-beta1	100	A**A**GTAA
-303	T/C	0.90	0.10	NF-AT	100	GG**T**TT
-324	A/C	0.91	0.09	AP-1	100	**GTCTCA**
				RXR-alpha	100	TC**A**CCC
-378	A/G	0.99	0.01	AP-4	100	**CAGCTG**
				PTF1-beta	87.5	CAGCT**G**TGC
				GR	100	CT**G**TACA
-468	C/T	0.90	0.10	-	-	-
-549	G/A	0.99	0.01	C/EBPalpha	87.5	AT**G**TTGCAA
-618	C/A	0.99	0.01	RXR-alpha	100	**A**GATTA
-681	C/T	0.91	0.09	CP2	100	**C**TGGAA
				C/EBPalpha	87.5	A**C**TGGAAAT
				STAT3	87.5	TAA**C**TGGAA
-698	A/G	0.91	0.09	TFIID	100	TTC**A**AA
-717	C/A	0.79	0.21	GR	100	T**C**TTAT
-742	C/A	0.68	0.32	NF-1	100	AGCC**A**A
-761	C/T	0.78	0.22	HES-1	100	CAC**C**AG
-790	C/G	0.71	0.29	NF-kappaB	87.5	AGGACA**C**CC
-862	G/C	0.62	0.38	-	-	-

(L663F) for the backfat thickness trait in exon 14 of the *LEPR* gene is significantly associated in multiple generations of an Iberian × Landrace intercross [20]. Mackowski et al. (2005) reported that three SNPs in exon 4 have no direct effect on fatness traits in Polish Landrace and the 990 synthetic line but that the A allele at locus 232T/A is significantly associated with thicker backfat over the shoulder in Polish Landrace [12]. Chen et al. (2004) reported that synonymous mutations of P300P in exon 6 and D947D in exon 18 are significantly associated with backfat thickness ($p<0.05$) in Landrace and Yorkshire, respectively, and that the effect of the SNP in exon 18 is significant for feed efficiency in Duroc [2]. However, no significant effect of the SNP variants on average daily weight gain was observed in the present study. Chen et al. (2004) reported that the associations among intron 2 and exons 2 and 18 polymorphisms and reproductive traits were significant in Duroc and Yorkshire [2]. In recently, a SNP (c.987C>T) of the porcine *LEPR* gene was significantly associated with feed intake, growth and fatness traits in pigs [15]. However, we did not find any effects of the exonic mutations on phenotypes. These inconsistent results might be due to the different samples used in this and previous studies.

Allelic frequencies and association analysis

Allelic frequencies of these exonic polymorphisms were investigated in nine different pig breeds (Table 3). These polymorphisms were present in almost all breeds, except for the T220A polymorphism in Duroc and the L243L polymorphism in the Korean wild pig and the Chinese breeds. The allelic frequency of each locus showed different patterns among the pig breeds. Half of all SNPs in the 5′ regulatory region were not informative in Duroc (Table 4). Dozens of transcription factor-binding sites were predicted in the 5′ regulatory region of the porcine *LEPR* gene. Among them, specificity protein 1 and C/EBP sites have been found in the human leptin gene promoter [7], and C/EBPa and PPARg modulate the expression of the human leptin gene [14, 21]. In addition, the transcription factors NFkB, liver X receptor, and hepatocyte nuclear factor-4α play important roles regulating gene expression of lipid metabolism [22].

A total of 550 pure Duroc pigs were genotyped on 24 SNPs including 6 SNPs on the exonic regions and 18 SNPs in the regulatory region for the association study. Only one SNP at the -790C/G polymorphism on the regulatory region in the *LEPR* gene was significantly associated with production traits such as backfat thickness ($p<0.001$) and lean

Table 5. Effect of the mutation (-790C/G) on productive traits in Duroc breed

Traits[a]	GG (n = 53)[b]	GC (n = 222)[b]	CC (n = 275)[b]	P value[c]
ADG (g/day)	1108.1±12.7	1107.4±10.3	1108.0±10.2	0.9932
FE	2.28±0.028	2.25±0.02	2.26±0.02	0.4196
BFT (mm)	1.47[a]±0.04	1.41[b]±0.03	1.38[b]±0.03	0.0019
LMP (%)	54.8[a]±0.8	55.7[b]±0.5	56.1[b]±0.5	0.0038

[a]ADG; average daily gain, FE; feed efficiency, BFT; back fat thickness, LMP; lean meat percentage
[b]Least square means with different letters indicate were different with statistical significance (p<0.05)
[c]Type I error rate whether the three marker genotypes have the same effects

meat percentage (p<0.003), but had no significant effect on average daily weight gain or feed efficiency (Table 5). That is, backfat thickness was higher and lean meat percentage lower in the individual of the genotype GG rather than in that of the genotype CC. However, no other significant associations of genotypes for the other 24 SNPs were found for the other traits.

The -790C/G polymorphism site was generated within the binding site AGGACAC/GCC) of the putative NF-kB transcription factor. NF-kB is involved in regulating gene expression as a transcription factor. This suggests that a polymorphism in the promoter region of the *LEP*R gene might be critical for binding a transcription factor such as NF-kB. A significant phenotypic effect may have been observed if a causal mutation in the LEPR promoter region occurred or if the mutation was closely linked with a causal mutation. Therefore, further studies are needed to determine whether these results are due to a polymorphic site that is critical for transcription or linkage disequilibrium with a causal mutation.

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초록 : 두록 돼지의 등지방두께와 연관된 렙틴수용체 유전자의 신규 SNP 마커

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돼지에게 있어서 지방 형질은 가장 중요한 경제형질 중 하나다. 돼지의 렙틴수용체 유전자(*LEPR*)는 염색체 상의 위치와 그 생리 활성 측면에서 돼지 6번 염색체 상의 지방형질 관련 양적형질좌위(QTL)에 대한 주요 후보유전자로 알려져 있다. 본 연구에서는 *LEPR* 유전자의 구조 변이와 돼지 경제형질과의 연관성을 분석하였다. 이를 위하여 돼지 *LEPR* 유전자를 포함하고 있는 박테리아 인공 염색체(BAC) 클론에 대한 샷건 염기서열 해독을 수행하여 114 kb 크기의 유전체 서열을 확보하였다. 그리고 전사개시 코돈으로부터 1.2 kb 상위 영역에서 여러 전사인자 결합부위를 발견하였다. 또한 *LEPR* 유전자 엑손 영역의 6개 SNP와 5′ 조절영역의 18개 SNP에 대해 550두의 두록 개체를 대상으로 연관성 분석을 수행하였다. 이들 SNP 중, -790C/G만이 등지방두께와 정육율 형질과 유의적으로 연관되어 있었으며, 2개의 미스센스 다형성 SNP를 포함하여 다른 SNP에서는 어떤 형질과도 연관성을 보이지 않았다. 결론적으로 -790C/G SNP는 두록 돼지에서 지방과 정육형질을 유전적으로 개량하는데 유용한 마커로 활용될 수 있을 것이다.