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A missense variant of the porcine melanocortin-4 receptor *(MC4R)* gene is associated with fatness, growth, and feed intake traits

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Abstract. Our knowledge of the genetic factors affecting obesity is increasing, but information about the individual gene effects remains limited in humans as well as in animal models. The melanocortin-4 receptor gene (*MC4R*) has been implicated in the regulation of feeding behavior and body weight in humans and mice. We have studied *MC4R* as a candidate gene for the control of economically important growth and performance traits in the pig. A missense mutation was identified in a region highly conserved among melanocortin receptor (MCR) genes. To determine whether there was an association of this *MC4R* polymorphism with phenotypic variation, we tested the mutation in a large number of individual animals from several different pig lines. Analyses of growth and performance test records showed significant associations of *MC4R* genotypes with backfat and growth rate in a number of lines as well as feed intake overall. It is probable that the variant amino acid residue of the *MC4R* mutation (or a closely linked mutation) causes a significant change of the *MC4R* function. These results support the functional significance of a pig *MC4R* missense mutation and suggest that comparative genomics based on model species may be equally important for application to farm animals as they are for human medicine.

Introduction

Maintenance of body weight is controlled by a sophisticated system that involves measurement of energy intake, external environmental conditions, and feedback, which then regulates further feed intake and expenditure. Therefore, numerous behavioral, environmental, and genetic factors can cause obesity. Identification of mutations in the leptin and the leptin receptor has provided some information on genetic components involved in the regulation of energy balance (Zhang et al. 1994; Tartaglia et al. 1995). Genetic studies with animal models have facilitated the identification of major genetic causes of obesity (Andersson 1996; Pomp 1997; Giridharan 1998). Furthermore, several other genes involved in the neural signaling pathway of energy homeostasis have been identified (Flier and Maratos-Flier 1998; Schwartz et al. 1999). Of particular interest among candidate signaling molecules involved in the regulation of energy homeostasis is the melanocortin-4 receptor (*MC4R*). The *MC4R* response to leptin signaling is a link between food intake and body weight (Seeley et al. 1997; Marsh et al. 1999). It is also believed that Neuropeptide Y (NPY) signaling in the central nervous system may be in parallel to that of *MC4R* signaling (Hahn et al. 1998). Several mutations in *MC4R*, including frameshift and nonsense mutations, are associated with dominantly inherited obesity in humans (Vaisse et al. 1998; Yeo et al. 1998). Some other *MC4R* missense mutations in humans have also been identified (Gotoda et al. 1997; Hinney et al. 1999), but the functional significance of these mutations has not been characterized.

Selection based on growth characteristics has been of great importance to the pig industry because of costs associated with feeding and consumer preference for lean meat. Efficient genetic improvement in these quantitative traits may be augmented through the use of marker-assisted selection (MAS) by use of high-density genetic maps (Dekkers and van Arendonk 1998; Rothschild and Plastow 1999). An important tool in this process is comparative mapping with the well-developed human and mouse gene maps, which assist in the identification of corresponding genomic regions or major genes controlling growth and performance traits in the pig. Biological understanding of complex traits in human or model species offers an alternative approach to identify genes responsible for the traits of economic interest in livestock. Several quantitative trait loci (QTL) linkage scans with phenotypically divergent breeds and candidate gene analyses have been successfully conducted for fatness and growth traits (Andersson et al. 1994; Yu et al. 1995; Casas-Carrillo et al. 1997; Knorr et al. 1997; Knott et al. 1998; Rohrer and Keele 1998; Wang et al. 1998; Paszek et al. 1999), but only a limited set of individual genes with major effects on growth and performance traits have been reported for commercial populations (Fuji et al. 1991; Jeon et al. 1999; Nezer et al. 1999). The role of *MC4R* in feed intake and obesity suggests it may be an important genetic marker for the growth-related traits in the pig. This paper presents strong evidence of associations between a porcine *MC4R* missense mutation and several obesity-related traits in pig, such as fatness, growth rate, and feed intake and discusses the usefulness of an *MC4R* gene test as a genetic tool in pig production. The missense polymorphism of porcine *MC4R* could also provide new insight into the major structural elements controlling melanocortin receptor functions.

Materials and methods

Animals. Pigs were raised under normal production conditions under the care of PIC employees in nucleus farms in the United States and Europe. Data and samples were collected from five different commercial lines of pigs (Table 1). Lines A and B were based on Landrace and Large White populations respectively and were established more than 30 years ago. Line C is a synthetic between Large White and Duroc populations. It was created approximately 8 years before the study. Line D is a synthetic line created by crossing several different synthetic populations at least 4 years prior to the study. Line E is a synthetic line derived by crossing Meishan and Large White populations approximately 10 years prior to the study. Pigs were put on the performance test at approximately 70 days of age and taken off test after 13 weeks. At the end of the trial, backfat was measured

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Table 1. Effect of *MC4R* genotype on several production traits in the pig.[a]

Number of observations (males/females/total) for days to 110 kg and backfat

MC4R	LINE A	LINE B	LINE C	LINE D	Total	LINE E
11	9/212/221	12/94/106		37/17/54	58/323/381	0/20/20
12	9/150/159	37/96/133	12/158/170	152/30/182	210/434/644	0/67/67
22	3/16/19	28/36/64	89/356/445	155/12/167	275/420/695	0/37/37

Days to 110kg

MC4R	LINE A	LINE B	LINE C	LINE D	Overall	LINE E
11	166.3 ± 0.8	168.4 ± 1.4		170.0 ± 2.4	167.9 ± 0.9	219.1 ± 4.8
12	165.6 ± 0.9	166.8 ± 1.1	163.9 ± 1.0	170.2 ± 1.8	166.9 ± 0.8	212.2 ± 3.4
22	162.3 ± 2.3	166.8 ± 1.5	161.5 ± 0.8	167.0 ± 1.9	164.6 ± 0.9	211.4 ± 4.0
	$P < .24$	$P < .47$	$P < .007$	$P < .10$	$P < .001$	$P < .27$

10^{th}rib backfat (mm)

MC4R	LINE A	LINE B	LINE C	LINE D	Overall	LINE E
11	10.7 ± 0.2	12.1 ± 0.2		9.8 ± 0.5	11.1 ± 0.2	22.8 ± 1.2
12	11.2 ± 0.2	12.5 ± 0.2	12.3 ± 0.2	10.5 ± 0.4	11.6 ± 0.2	21.5 ± 0.9
22	12.5 ± 0.5	12.6 ± 0.3	12.7 ± 0.2	10.9 ± 0.4	12.0 ± 0.2	20.3 ± 1.0
	$P < .02$	$P < .31$	$P < .06$	$P < .05$	$P < .001$	$P < .17$

Number of observations (males/females/totals) for test daily gain

MC4R	LINE A	LINE B	LINE C	LINE D	Total	LINE E
11	9/105/114	12/38/50		37/17/54	58/160/218	0/20/20
12	9/65/74	37/35/72	12/97/109	152/30/182	210/227/437	0/67/67
22	3/13/15	28/15/43	89/225/314	155/12/167	275/265/539	0/37/37

Test daily gain (gm/day)

MC4R	LINE A	LINE B	LINE C	LINE D	Overall	LINE E
11	892.6 ± 10.4	841.7 ± 13.8		882.2 ± 18.4	871.9 ± 10.2	688.8 ± 24.5
12	913.3 ± 11.6	868.4 ± 12.1	882.2 ± 12.9	883.7 ± 14.3	885.1 ± 8.9	676.2 ± 17.6
22	982.8 ± 22.8	862.4 ± 15.1	913.4 ± 10.5	904.6 ± 15.1	908.8 ± 9.3	692.5 ± 20.4
	$P < .001$	$P < .28$	$P < .006$	$P < .20$	$P < .001$	$P < .66$

Number of observations (males/females/total) for average daily feed intake

MC4R	LINE A	LINE B	LINE C	LINE D	Overall	LINE E
11	7/0/7	11/0/11		13/0/13	31/0/31	0/18/18
12	8/0/8	31/0/31	9/0/9	34/0/34	82/0/82	0/63/63
22	3/0/3	25/0/25	74/0/74	16/0/16	118/0/118	0/32/32

Average daily feed intake (kg/day), boars only except LINE E, which was gilts only

MC4R	LINE A	LINE B	LINE C	LIND D	Overall	LINE E
11	2.31 ± 0.2	1.78 ± 0.09		1.75 ± 0.08	1.94 ± 0.07	2.05 ± 0.10
12	2.11 ± 0.3	1.90 ± 0.07	1.97 ± 0.10	1.90 ± 0.07	2.03 ± 0.06	2.03 ± 0.07
22	2.15 ± 0.4	1.97 ± 0.06	2.00 ± 0.07	1.97 ± 0.08	2.11 ± 0.06	2.08 ± 0.08
	$P < .84$	$P < .14$	$P < .56$	$P < .14$	$P < .01$	$P < .36$

[a] Line A is a Landrace-based population. Line B is a Large White-based population. Line C is a synthetic population based on Duroc and Large White. Line D is a synthetic line based on several different populations including Landrace, Large White, Duroc, and Pietrain. Line E is a synthetic population based on Meishan and Large White.

ultrasonically in real time (B mode) at the 10^{th} rib 2 cm from the centerline. Average daily gain (growth) over the test period was calculated as weight gained divided by days on test. The number of days to 110-kg market weight was estimated by standard procedures, and feed intake was measured with individual electronic measurement equipment.

PCR amplification of a pig MC4R gene fragment. Primers were designed from homologous regions of human and rat *MC4R* sequences (GenBank accession no. s77415 and u67863, respectively). The primers were: forward primer, 5′-TGG CAA TAG CCA AGA ACA AG-3′; and reverse primer, 5′-CAG GGG ATA GCA ACA GAT GA-3′. The PCR reaction was performed with 12.5 ng of porcine genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0 → 0.125 mM dNTPs, 0.3 mM of each primer, and 0.35 U *Taq* DNA polymerase (Promega, Madison, WI) in a 10-μl final volume. The conditions for PCR were as follows: 2 min at 94°C; 35 cycles of 30 s at 94°C, 1 min at 56°C, 1 min 30 s at 72°C; and a final 15-min extension at 72°C in a Robocycler (Strategene, La Jolla, Calif.).

Sequencing and mutation detection. Sequencing of the PCR products from several individual pigs of different breeds was conducted, and the sequences were compared to detect any nucleotide change. Sequencing was performed on an ABI sequencer 377 (Applied Biosystems, Foster City, CA). The porcine *MC4R* sequence has been submitted to GenBank and has accession number AF087937. The sequence analysis revealed one nucleotide substitution situated within a *Taq*I restriction enzyme recognition site (Kim et al. 1999). A new set of primers was then designed to generate a smaller *MC4R* gene fragment, which contained only one informative *Taq*I restriction site to specify the polymorphic site and to facilitate the PCR-RFLP test. These primers were: forward, 5′-TAC CCT GAC CAT CTT GAT TG-3′; and reverse, 5′-ATA GCA ACA GAT GAT CTC TTT G-3′.

Statistical analyses. Analyses of variance procedures were used with a mixed model that accounted for the fixed effects of farm, test period, sex of the animal, the *MC4R* genotype, and sire (random). All animals in lines of American/European descent (Lines A–D) were pooled for the overall

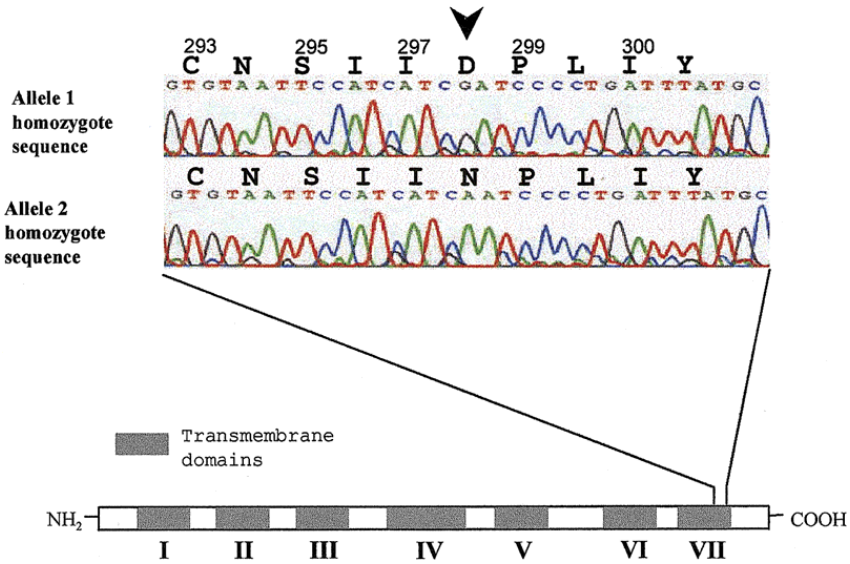

Fig. 1. Partial sequences of the porcine *MC4R* gene. The G→A substitution of the PCR product is marked with an arrow. The amino acid translation shows an amino acid substitution at codon 298. The schematic diagram represents the structure of MC4R and location of the mutation.

analysis, and in this analysis line of origin was included. Mean effects were estimated for each genotype and are presented in Table 1. Overall F tests were used to determine level of significance.

Results

Identification of a missense mutation in the pig MC4R gene. The *MC4R* gene consists of approximately 1 kb of coding sequence contained within a single exon. About 750 bp of a pig *MC4R* gene fragment were produced by PCR (Kim et al. 1999). The sequence of the PCR product confirmed that the PCR product is the *MC4R* gene with 92.2% and 97.6% identities at nucleotide and the amino acid levels, respectively, to the human *MC4R* sequence. Multiple alignments of the sequences from individual animals of several breeds identified a single nucleotide substitution (G→A; Fig. 1). The polymorphism revealed a missense mutation that replaces aspartic acid (GAU) with asparagine (AAU) at the position identical to amino acid 298 of human *MC4R* protein. To confirm this base change, we designed pig-specific primers flanking the polymorphic site and analyzed the polymorphism as a *Taq*I PCR-RFLP (Fig. 2).

The MC4R missense mutation is within a highly conserved region among melanocortin receptors (MCR). The MCR is a subfamily of G-protein coupled receptors (GPCR) containing certain conserved structural elements common to most other GPCRs, but overall amino acid identities between MCR and other GPCRs are low (Tatro 1996). A multiple alignment of the predicted amino acid sequences of the pig *MC4R* with *MC4R* proteins from other species, other MCR protein, or representative GPCRs showed that the aspartic acid found at position 298 of the seventh transmembrane domain is very highly conserved in the MCR proteins (Fig. 3). It is interesting to note, however, that this position is occupied by asparagine in most other GPCRs. The MCR proteins show 40–80% amino acid identity with each other (Tatro 1996), but the second intracytoplasmic loop and the seventh transmembrane domain are highly conserved among MCR proteins (Gantz et al. 1993). Some of the relationships between MCR structure and function have been discovered by the studies of natural and experimental mutations in humans and mice (Robbins et al. 1993; Valverde et al. 1995; Frandberg et al. 1997). These studies indicate that some mutations in highly conserved regions cause structural changes and alter the function of the receptor. The Asp298Asn substitution mutation could have an effect on the function of the receptor. This will require further testing, but it is known that change of the homologous residue in MC1R (Asp294His) is associated with fair skin and red hair in humans (Valverde et al. 1995).

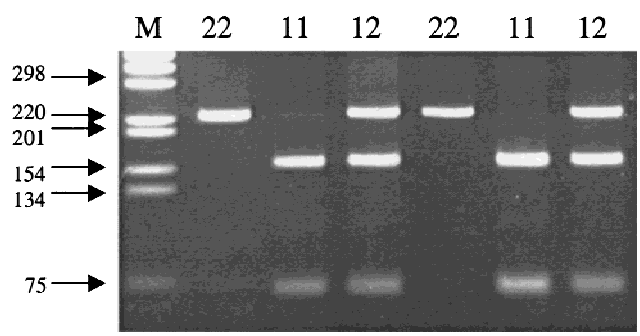

Fig. 2. *Taq*I digestion of the PCR product, analyzed by agarose-gel electrophoresis. Allele 1 produced 156- and 70-bp fragments, and allele 2 produced a 226-bp fragment as the PCR-RFLP. The heterozygote has both allele 1 and 2 fragments. Molecular marker (M) and *MC4R* genotypes are indicated at the top of each lane.

The MC4R missense mutation is associated with obesity-related traits. To investigate the effects of the missense mutation, we analyzed the relationship of *MC4R* genotypes for the effects on variation in growth rate, backfat, and feed consumed in over 1800 animals from several commercial pig lines from PIC, an international pig breeding company. The animals were from closed commercial lines of European/American breeds (lines A–D), together with a line originating from a cross between a European and a Chinese breed (line E). In lines A–D, significant association of the *MC4R* genotypes were found for all performance traits. In the overall analyses, the animals homozygous for allele 1 had on average significantly less backfat ($P < .001$), lower daily gain ($P < .001$), and lower feed intake ($P < .01$) than those of the homozygous 22 genotype animals (Table 1). Similar significant

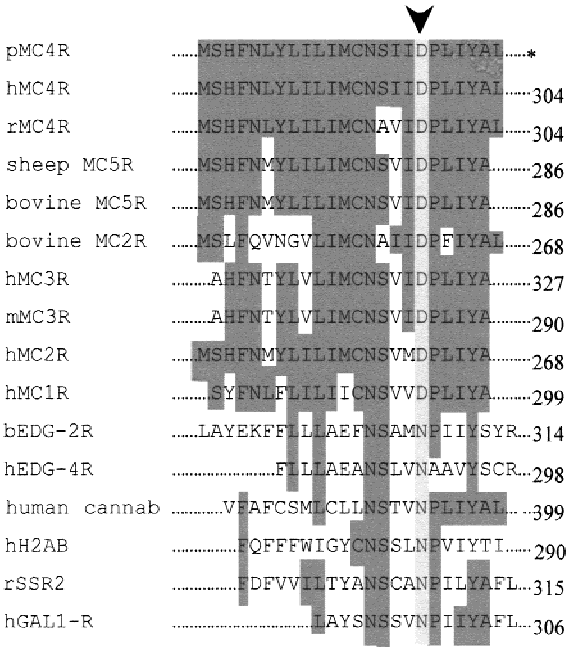

Fig. 3. Multiple alignments of the putative seventh transmembrane domain of porcine *MC4R* with other MCRs and GPCRs. The * represents the predicted sequence positions for porcine *MC4R*. The other amino acid sequences were obtained from the GenBank database (accession numbers P32245, P70596, P41983, P56451, P34974, P41968, P33033, Q01718, Q01726, Q28031, AF011466, P21554, P18089, P30680, P47211). The missense variant in porcine *MC4R* substituted amino acid N for D in the position marked with an arrow. The Asp (D) residue is highly conserved among MCR, and the Asn (N) residue is well conserved in most other GPCRs.

differences for backfat and growth were seen for most lines individually. Overall, pigs with the 11 genotype had approximately 9% less backfat than pigs with the 22 genotype, whereas pigs with the 22 genotype grow significantly faster (37 g/day) than pigs with the 11 genotype. These results appear to be a function of appetite, because the 22 genotype animals consume considerably more feed. The association between the missense variant of the *MC4R* gene and related performance traits is clearly established in European/American breeds. Interestingly, in some of the lines (A–D) the gene frequencies are not in Hardy-Weinberg equilibrium and may reflect selection pressure for one or another performance trait or that they are more recent synthetic lines. Although the number of tested animals is much smaller, these results were not seen in the considerably fatter Chinese crossed line (line E). Interestingly, line E shows a trend for backfat in the direction opposite to that observed in the other lines.

Discussion

The present study clearly demonstrates that the porcine *MC4R* missense mutation is significantly associated with several performance traits in pigs. Allele 1 representing Asp298, the well-conserved amino acid within other MCR subtypes and other species *MC4R*, was associated with less backfat thickness, slower growth rate, and lower feed intake, and allele 2, representing Asn298, was associated with fatter, higher-feed consuming, and faster-growing animals. As the highly conserved residues in the melanocortin receptor proteins have important roles for ligand binding or intracellular signal transmission (Tatro 1996), the *MC4R* variants might exert functionally distinct abilities in the regulation of food intake and body weight. Alternatively, the observed association could result from linkage disequilibrium between this polymorphism and the causative mutation (either in *MC4R* or in a tightly linked gene). This might explain the different results observed for Line E (see below). However, two of the lines (Lines A and B) in this study were established more than 30 years ago, and the most recent synthetic (Line D) was established 4 years ago. This, together with the fact that the effect is consistent among three completely unrelated lines (Lines A, B, and D), leads us to suggest that the most likely hypothesis is that the mutation observed is the causative mutation. Further testing of this hypothesis, including additional sequencing in other regions of the gene and functional studies, will provide important insights into the structural basis of the MCR function.

Allele 1 was associated with the fattest animals in line E, which was derived by crossing a Chinese (Meishan) breed with a line of Large White origin. This is surprising, given that the mutation causes a significant amino acid change in a well-conserved region and also since three unrelated lines (lines A, B, and D) appear to give the same phenotypic results. There is the possibility that the results in lines A–E are due to linkage disequilibrium between this mutation and the causative mutation, and this has eroded in line E and one or both of the founding populations. The line E result may also be due to sampling. However, if we assume that this result will be significant when more results are added, there are several possible explanations. One possibility could be the difference in the background gene effects (epistasis). Since growth and fatness are complex polygenic traits, it is certainly possible that the Chinese breed has some distinct allelic interactions derived from several hundred years of isolation, and these putative interaction(s) might create variation in polygenic traits within crosses between widely different lines (Frankel and Schork 1996). Several QTL analyses have been conducted for fatness and growth traits with divergent lines (Andersson et al. 1994; Casas-Carrillo et al. 1997; Knott et al. 1998; Rohrer and Keele 1998; Wang et al. 1998; Paszek et al. 1999), but QTLs have not been reported near the *MC4R* locus, which maps to Chromosome (Chr) 1 at approximately 80 cm (near S0313) on the linkage map (Kim et al. 1999). In fact the only QTL reported on Chr 1 is probably 50 cM distal to *MC4R* (Rohrer and Keele 1998). It may mean that the epistatic effects of the *MC4R* alleles suggested in line E have made it difficult to observe the *MC4R* locus in most QTL experiments that have involved crosses between Chinese and European/American lines. It is likely that the effect of some alleles will be variable in the different backgrounds and hard to detect in QTL experiments involving genetically divergent breeds.

The effect of the *MC4R* variant will possibly be explained by further studies on the biological effect caused by this mutation in other pig breeds and lines. However, given the strong relationship of *MC4R* variants to leanness, growth, and feed intake, this mutation could be used immediately for marker-assisted selection (Meuwissen and Goddard 1996) to develop lines of pigs to satisfy particular customer requirements. For instance, in sow lines where appetite is normally decreased after farrowing, selection for the *MC4R* allele 2 could help to improve feed intake. Furthermore, in some lines deemed to be too fat, selection for allele 1 could be employed, and in lines that were considered to be growing too slowly, allele 2 selection could be employed. Therefore, genotyping for the *MC4R* mutation in pig breeding lines will improve the selection efficiency of feed-related production traits including growth and leanness.

The candidate gene approach has also been used for investigating the role of the porcine leptin gene (Jiang and Gibson 1999). However, in the leptin case, although there was some evidence for an association between a leptin polymorphism and backfat depth in one (of four) commercial lines of pigs tested, this result was not confirmed by re-sampling of the same population (Jiang and Gibson 1999). A study of the same polymorphism to analyze commercial lines of pigs in Germany also failed to find an association (T. Hardge and coworkers, personal communication). In contrast, with *MC4R* we have determined that variation in this candidate gene can explain significant variation for backfat, growth rate, and

feed intake in commercial lines of pigs. These results with *MC4R* illustrate the potential value of comparative genetic analyses with candidate genes in livestock genomics.

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