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Detailed characterization of the porcine *MC4R* gene in relation to fatness and growth

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Summary

In contrast to the human *MC4R* gene, where multiple variants have been described, several of which are associated with appetite and obesity, few *MC4R* variants have been reported in the pig. The most interesting polymorphism reported to date in the pig is p.Asp298Asn, which is significantly associated with variation in growth and fatness traits in most breeds and crosses. However, some reports have seemingly failed to confirm this association. The discrepancy of p.Asp298Asn associations in some pig populations suggested that further discovery of SNPs in *MC4R* would be useful. Utilizing the recently released pig genome sequence information, we obtained the whole *MC4R* genome sequence and detected five additional SNPs, a variable $(CA)_n$ repeat and a C indel in the ISU Berkshire × Yorkshire pig resource family. Linkage disequilibrium (LD) analysis revealed that the additional five SNPs were not in strong LD with p.Asp298Asn, but single marker association analysis indicated that they were significantly ($P < 0.05$) associated with fatness measures and very highly significantly ($P < 0.0001$) associated with average daily gain on test (ADGTEST). Three major haplotypes were identified and the subsequent association analyses suggested that the two non-synonymous SNPs had different effects, e.g. p.Arg236His influenced back fat and growth on test while p.Asp298Asn was primarily associated with variation in growth rate in this population. An interaction effect between these two SNPs was found for ADGTEST, which may partly explain some of the previous discrepancies reported for *MC4R* in different pig populations. Examination of the p.Arg236His polymorphism in populations where the effect of p.Asp298Asn is limited is warranted.

Keywords association, haplotype, *melanocortin-4 receptor*, pig.

Introduction

The melanocortin-4 receptor (MC4R), a Rhodopsin-like G protein-coupled receptor, is primarily expressed in the nervous system and plays an important role in the regulation of food intake, energy balance and body weight in mammals (Govaerts *et al.* 2005; Adan *et al.* 2006). *MC4R* gene variants in humans are associated with early onset or severe adult obesity (Lubrano-Berthelier *et al.* 2003a). *In vivo* functional analysis revealed that some of these variants could reduce cAMP response and cell surface expression, and displayed partially or totally intracellular retained effects (Hinney *et al.* 2006; Stäubert *et al.* 2007). Most recently, two independent genome-wide association studies indicated that several common variants near *MC4R* were significantly associated with fatness, body weight and obesity pre-disposition (Chambers *et al.* 2008; Loos *et al.* 2008). The disruption of *MC4R* in mice caused an obese phenotype (Huszar *et al.* 1997), and *MC4R* knock-out mice had more obvious characteristics of maturity-onset obesity than wild types (Ste Marie *et al.* 2000). In pigs, a missense mutation, p.Asp298Asn was found to be associated with fatness and daily gain, and its impact on the stimulation of cAMP production has been demonstrated (Kim *et al.* 2000a, 2004a; Barb *et al.* 2004). Significant associations of

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p.Asp298Asn with fatness and growth rate traits were reported in a large number of pig populations with various genetic backgrounds (Hernández-Sánchez *et al.* 2003; Houston *et al.* 2004; Bruun *et al.* 2006; Jokubka *et al.* 2006; Meidtner *et al.* 2006; Óvilo *et al.* 2006; Van den Maagdenberg *et al.* 2007; D'Andrea *et al.* 2008), but it could not be validated in some other populations, although these studies were of relatively small size (Park *et al.* 2002; Stachowiak *et al.* 2005). *MC4R* was mapped to 1q22 on SSC1 (Kim *et al.* 2000b). Preliminary sequence annotations of several chromosomes including SSC1 have now been released as part of the effort to sequence the swine genome (http://pre.ensembl.org/Sus_scrofa/index.html), which facilitates an analysis of the whole genomic sequence of *MC4R*. Therefore, the aim of this study was to identify additional variation in the porcine *MC4R* gene, and to carry out haplotype construction and association analysis so as to contribute to the understanding of the role of *MC4R* in variation of growth and fatness traits in pigs.

Materials and methods

Animals and traits

DNA samples were obtained from the ISU Berkshire and Yorkshire (B × Y) intercross breed resource family, previously used for QTL mapping for growth, carcass composition and meat quality traits (Malek *et al.* 2001). Briefly, two F_0 Berkshire sires were crossed with nine F_0 Yorkshire dams to produce nine F_1 litters, and then eight sires and 26 dams from these F_1 litters were selected and crossed to produce 515 F_2 animals.

The traits analysed in the study comprise the four fatness traits: average back fat; back fat on the sites of the lumbar, 10th rib and last rib; and two growth rate traits: average daily gain from birth to weaning (ADGW) and average daily gain on test (ADGTEST). The details of these trait measurements have been described by Malek *et al.* (2001).

MC4R variants discovery and genotyping

Human *MC4R* transcript sequence (Ensemble transcript ID: ENST00000299766) retrieved from Ensemble database (http://www.ensembl.org/index.html) was used as an entry to search for homologous pig sequence (http://www.ncbi.nlm.nih.gov/projects/genome/guide/pig). Two pig genome sequence fragments from clone CH242-399A6 (CU618334.2) of chromosome 1, showing 92% and 86% identity with the entry sequence respectively were obtained. Four pairs of primers (1F: GTGGCGAAGGTCACAATGG/1R:AGTGGCTCCTCCTCTGCTT; 2F:TCTTCTCCCAATAGCACAGC/2R:GGAAACGCTCACCAGCATA; 3F:GGTGTTTGTGACTCTGGGTGT/3R:GATTCTGGGGGCAGGAGATA; 4F: TCCCCTGATTTATGCACTCC/4R:TTTCTCCCCTCAATGCTAGA) were designed for PCR amplification of *MC4R* gene from pig genomic DNA. PCR products were commercially sequenced. The sequences were integrated into one whole sequence using the ensemble option of SEQUENCHER software 4.6 (Gene Codes), which showed 99% similarity with the blasted pig genome sequence. The ensemble sequence has been deposited into GenBank of NCBI (accession number: FJ357500).

In an effort to discover SNPs in a cost-effective manner, SNP discovery was implemented by sequencing pooled PCR products, which were amplified from DNA of founder animals from the ISU B × Y resource family. The sequences were imported into the SEQUENCHER software 4.6 (Gene Codes) and searched for SNPs. PCR primers were redesigned to facilitate genotyping of the additional variants using PCR-RFLP technique in the B × Y population.

Association analyses of single markers and haplotypes

The association analyses between single SNPs and fatness and growth rate traits were performed with a statistical model using the MIXED model procedures (SAS 9.0; SAS Institute). In this model, sex, slaughter date and marker genotypes were considered as fixed effects, dam (litter) as a random effect and carcass body weight as a covariate.

Haplotype analyses and graphical representation of the linkage disequilibrium (LD) structure as measured by r^2 were performed with the HAPLOVIEW software (Ver 3.32) (Barrett *et al.* 2005). Haplotypes were obtained for each animal using the PHASE computer program (Ver 2.1) (Stephens *et al.* 2001). The association analyses between different copy numbers of certain haplotypes and traits were implemented using the MIXED procedure of SAS as mentioned above.

Combined association analyses were carried out to explore the possible interaction between the SNPs. The model was similar to that of single marker association analysis, except that the interaction between the two SNPs was included as a fixed effect.

Linkage mapping and QTL analysis

Based on the genotypes of the p.Asp298Asn SNP, pig *MC4R* has been mapped between *S0331* and *Sw974* on SSC1 (Kim *et al.* 2000b). Linkage mapping of additional SNPs identified in the study was performed with two-point linkage analyses using an improved CRIMAP (Ver 2.5, Green *et al.* 1990) developed by Evans I & Maddox J (personal communication). The QTL analyses were performed using online GridQTL (http://www.gridqtl.org.uk/; Seaton *et al.* 2006). The impact of *MC4R* was determined initially by including the *MC4R* haplotype in the model as additional marker. Subsequently, the *MC4R* genotypes and haplotypes were included as fixed effects. In the model, sex and slaughter year were used as fixed effects and carcass body weight as a covariate.

Results

Additional variants identified in pig *MC4R* gene

In total, eight variants including a $(CA)_n$ repeat variant, a C indel and six SNPs were identified in the study (Table 1). Of four variations within the proximal promoter and 5′ UTR, the SNPs c.-780C>G and c.-135C>T could cause disruption of several transcription factor binding sites (TFBS), but c.-746CA(6_7) and c.-702delC would not, as predicted by TESS online (http://www.cbil.upenn.edu/cgi-bin/tess/tess). The SNP c.175C>T is a synonymous mutation p.Leu59Leu, while SNPs c.707A>G and c.892A>G resulted in non-synonymous mutations p.Arg236His and p.Asp298Asn respectively. The SNP c.*430A>T within the putative 3′ UTR was not found to be a target region for microRNA binding using the Patrocles finder (http://www.patrocles.org). The three exonic SNPs p.Leu59Leu, p.Arg236His and p.Asp298Asn have been found in previous studies, but the occurrences of the former two were very rare (Kim *et al.* 2004b; Meidtner *et al.* 2006; Óvilo *et al.* 2006). According to the structural conservation of amino acid positions in human *MC4R* orthologues (Stäubert *et al.* 2007), p.Asp298Asn is located in the transmembrane domain 7, and p.Arg236His is in intracellular loop 3. All of the above variants have been deposited to dbSNP of NCBI (accession number: ss107793981–ss107793988).

Association analysis of single markers and haplotypes

The genotyping of the five additional SNPs and p.Asp298Asn was successfully implemented using PCR-RFLP in the ISU B × Y pig population (Table 1). All SNPs were in Hardy–Weinberg equilibrium except p.Asp298Asn, and each had a minor allele frequency greater than 0.05. The results of the association analyses between single markers and the fatness and growth rate traits are shown in Table S1 and Fig. 1a. The SNP p.Asp298Asn was highly significantly associated ($P < 0.01$) with ADGW and ADGTEST, significantly associated ($P < 0.05$) with back fat thickness at 10th rib, and suggestively associated ($P < 0.10$) with average back fat and lumbar back fat. The other five SNPs had highly significant associations ($P < 0.01$) with average back fat, back fat at the lumbar and last rib, and with ADGTEST, while none of them were associated with 10th rib back fat and ADGW. They were also suggestively ($P < 0.1$) or closely suggestively associated (p.Arg236His, $P < 0.12$) with loin eye area (LEA). Interestingly, the results for these five SNPs were very similar for all traits and were clearly different from p.Asp298Asn (Fig. 1a).

Haplotype analyses and graphical representation of LD structure are shown in Fig. 1b. As expected from the individual SNP association results, SNPs c.-780C>G, c.-135C>T, p.Leu59Leu, p.Arg236His and c.*430A>T had

Table 1 Genetic variants identified in the *MC4R* gene in the ISU Berkshire × Yorkshire pig resource family.

No.	Variant type	Variant location	Possible genetic effect	Primer sequences F/R (5′ → 3′)	Tm (°C)	Restriction enzyme	PCR-RFLP pattern (bp)	MAF	HW *P*-value	dbSNP accession no.
1	c.-780C>G[1]	Proximal promoter	TFBS disruption (TCF1, TCF2α, LEF1 and DOFs)	GTGGCGAAGGTCACAATGG/AGTGGCTCCTCCTCTGCTT	60	BglI	640/338 + 302	0.270	0.169	107793981
2	c.-746CA(6_7)[1]	Proximal promoter	–	–	–	–	–	–	–	107793987
3	c.-702delC[1]	Proximal promoter	–	–	–	–	–	–	–	107793988
4	c.-135C>T[1]	5′ UTR	TFBS disruption (SEF1, ETF, SGF1 and TFIID)	TCTTCTCCCAATAGCACAGC/GGAAACGCTCACCAGCATA	60	Tsp45I	401 + 135/292 + 209 + 135	0.269	0.182	107793982
5	c.175C>T	Exon 1	p.Leu59Leu	CAGGTCAGAGGGGATCTCAA/GTGCAGACTGCCCAGATACA[2]	62	HindIII	568/206 + 362	0.266	0.112	107793983
6	c.707A>G	Exon 1	p.Arg236His	TCGATTGCAGTGGACAGGTA/GAAAATGCTGTTGTTGAAGCA[2]	62	MwoI	464 + 199/382 + 199 + 82	0.268	0.123	107793984
7	c.892A>G	Exon 1	p.Asp298Asn	TCGATTGCAGTGGACAGGTA/GAAAATGCTGTTGTTGAAGCA[2]	60	TaqI	662 + 1/466 + 196 + 1	0.457	<0.001	107793985
8	c.*430A>T[1]	3′ UTR	–	AATGGGGACAGAGGAGACTT/CTGCACAGGGAGAATGAGC	62	NlaIII	481 + 18/429 + 52 + 18	0.267	0.109	107793986

[1]SNPs newly identified in this study.
[2]Obtained from Meidtner *et al.* (2006).

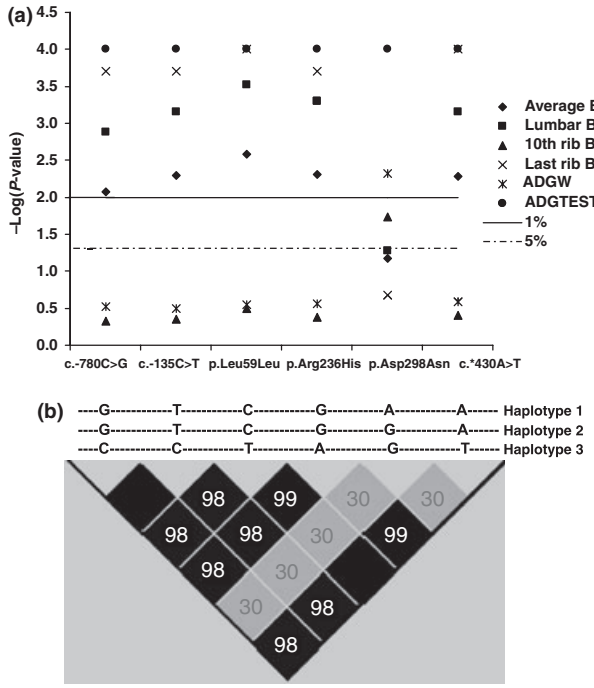

Figure 1 Single marker associations and haplotype construction for six SNPs in the *MC4R* gene. (a) Marker associations: the *x*-axis indicates SNP identity and the *y*-axis indicates the significance level (see Table S1; −Log(*P*-value) is a transformed value based on the raw *P*-value). Fatness and growth traits are described as in Table 2. (b) Linkage disequilibrium: Black boxes indicate r^2 values between 0.8 and 1.0, and light boxes indicate r^2 values less than 0.80.

strong and highly significant LD between each other ($r^2 \geq 0.98$; Fig. 1b), while p.Asp298Asn did not display high LD with them, although it is located between p.Arg236His and c.*430A>T. Three major haplotypes accounting for 99.9% of the alleles were obtained as follows, haplotype 1, -GTCGAA- (45.6%), haplotype 2, -GTCGGA- (27.4%) and haplotype 3, -CCTAGT- (26.7%). The results of association analyses between haplotype combinations and traits are shown in Table 2. There were significant differences ($P \leq 0.05$) between haplotype combinations on all of the analysed traits. There was a significant or suggestively significant ($P < 0.1$) difference between individuals carrying haplotype 1 and those without haplotype 1 for all of traits, except last rib back fat. The presence of haplotype 1 favoured ADGTEST with a very high significance ($P < 0.0001$). Haplotype 3, showed significant ($P < 0.05$) associations with all of traits except 10th rib back fat and ADGW. Haplotype 2 was associated with fatness traits but not growth.

The combined association results for p.Arg236His and p.Asp298Asn are shown in Table 3. The p.Asp298Asn polymorphism had significant effects on average back fat, 10th rib back fat and ADGW, and was suggestively significant for ADGTEST and lumbar back fat ($P < 0.10$). The p.Arg236His polymorphism had a significant effect on ADGTEST and all fatness traits except 10th rib back

Table 2 The associations of *MC4R* haplotype combinations and copy numbers of specific haplotype with fatness and growth rate traits respectively.

	LSM (SE)[1] of haplotype combination							LSM (SE) of copies of haplotype 1 -GTCGAA-				LSM (SE) of copies of haplotype 2 -GTCGGA-				LSM (SE) of copies of haplotype 3 -CCTAGT-			
Trait	11	12	13	22	23	33	*P*-value	0	1	2	*P*-value	0	1	2	*P*-value	0	1	2	*P*-value
Average BF	3.18^{a}	3.22^{a}	3.41^{b}	3.12^{a}	3.20^{a}	3.34^{ab}	0.003	3.21^{a}	3.31^{b}	3.19^{a}	0.070	3.33^{a}	3.21^{b}	3.12^{b}	0.009	3.19^{a}	3.34^{b}	3.36^{ab}	0.004
	(0.07)	(0.06)	(0.06)	(0.10)	(0.08)	(0.11)		(0.06)	(0.05)	(0.07)		(0.05)	(0.05)	(0.10)		(0.05)	(0.05)	(0.10)	
Lumbar BF	3.41^{a}	3.47^{a}	3.73^{b}	3.39^{a}	3.51^{a}	3.61^{ab}	0.009	3.49^{a}	3.60^{a}	3.41^{b}	0.049	3.62^{a}	3.48^{b}	3.38^{b}	0.016	3.44^{a}	3.66^{b}	3.61^{b}	0.006
	(0.09)	(0.07)	(0.07)	(0.12)	(0.09)	(0.13)		(0.08)	(0.06)	(0.09)		(0.06)	(0.06)	(0.12)		(0.06)	(0.07)	(0.13)	
10th rib BF	3.10^{ab}	3.09^{ab}	3.22^{a}	2.95^{b}	2.84^{b}	3.06^{ab}	0.047	2.96^{a}	3.16^{b}	3.10^{ab}	0.024	3.16^{a}	3.05^{b}	2.96^{b}	0.060	3.07	3.14	3.06	0.475
	(0.09)	(0.07)	(0.07)	(0.12)	(0.12)	(0.13)		(0.08)	(0.06)	(0.09)		(0.06)	(0.06)	(0.01)		(0.06)	(0.07)	(0.13)	
Last rib BF	3.03^{a}	3.07^{a}	3.26^{b}	3.02^{a}	3.15^{ab}	3.37^{b}	0.001	3.17	3.17	3.04	0.173	3.21^{a}	3.09^{b}	3.02^{b}	0.024	3.05^{a}	3.23^{b}	3.38^{b}	0.0001
	(0.07)	(0.05)	(0.06)	(0.06)	(0.08)	(0.11)		(0.06)	(0.05)	(0.07)		(0.05)	(0.05)	(0.10)		(0.05)	(0.05)	(0.11)	
ADGW	0.26^{a}	0.23^{b}	0.23^{b}	0.23^{b}	0.23^{b}	0.22^{b}	0.056	0.23^{a}	0.23^{ab}	0.26^{b}	0.006	0.23	0.23	0.23	0.624	0.24	0.23	0.22	0.247
	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)		(0.01)	(0.01)	(0.01)		(0.01)	(0.01)	(0.01)		(0.01)	(0.01)	(0.01)	
ADG TEST	0.71^{a}	0.69^{b}	0.68^{b}	0.70^{ab}	0.67^{b}	0.64^{c}	<0.0001	0.68^{a}	0.69^{ab}	0.71^{b}	<0.0001	0.68 (0.06)	0.68 (0.01)	0.70 (0.10)	0.115	0.70^{a}	0.68^{b}	0.64^{c}	<0.0001
	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)		(0.01)	(0.01)	(0.01)						(0.01)	(0.01)	(0.01)	

Average BF, average back fat thickness on three sites; Lumbar BF, back fat at last lumbar; 10th rib BF, back fat at the 10th rib; Last rib BF, back fat at last rib; ADGW, average daily gain from birth to weaning; ADGTEST, average daily gain on test.

[1]LSM (SE) represents least squares means and their standard errors. Superscripts a, b and/or c differ significantly ($P < 0.05$) from each other.

Table 3 Least squares means for combined association analysis of p.Arg236His and p.Asp298Asn on fatness and growth rate traits in the ISU Berkshire × Yorkshire pig resource family.

			p.Asp298Asn		
Trait	SNP		AA	AG	GG
Average BF		AA	–	–[1]	3.33 (0.11), *n* = 30
	p.Arg236His	AG	–	3.41 (0.06), *n* = 152	3.18 (0.08), *n* = 61
		GG	3.19 (0.07), *n* = 75	3.21 (0.06), *n* = 150	3.11 (0.09), *n* = 36
		P-value	p.Arg236His, <0.05	p.Asp298Asn, <0.05	Interaction, >0.10
Last rib BF		AA	–	–	3.38 (0.11), *n* = 30
	p.Arg236His	AG	–	3.26 (0.06), *n* = 152	3.12 (0.08), *n* = 61
		GG	3.04 (0,07), *n* = 75	3.05 (0.06), *n* = 150	3.02 (0.09), *n* = 36
		P-value	p.Arg236His, <0.01	p.Asp298Asn, >0.10	Interaction, >0.10
Lumbar BF		AA	–	–	3.59 (0.13), *n* = 30
	p.Arg236His	AG	–	3.74 (0.07), *n* = 152	3.47 (0.09), *n* = 61
		GG	3.43 (0.09), *n* = 75	3.45 (0.07), *n* = 150	3.38 (0.12), *n* = 36
		P-value	p.Arg236His, <0.05	p.Asp298Asn, <0.10	Interaction, >0.10
10th rib BF		AA	–	–	3.02 (0.12), *n* = 30
	p.Arg236His	AG	–	3.23 (0.07), *n* = 152	2.94 (0.09), *n* = 61
		GG	3.11 (0.09), *n* = 75	3.11 (0.07), *n* = 150	2.96 (0.11), n=36
		P-value	p.Arg236His, >0.10	p.Asp298Asn, <0.05	Interaction, >0.10
LEA	p.Arg236His	AA	–	–	35.84 (0.99), *n* = 30
		AG	–	35.29 (0.64), *n* = 152	35.95 (0.78), *n* = 61
		GG	35.47 (0.74), *n* = 75	36.69 (0.64), *n* = 150	36.35 (0.92), *n* = 36
		P-value	p.Arg236His, >0.10	p.Asp298Asn, >0.10	Interaction, >0.10
ADGW		AA	–	–	0.22 (0.01), *n* = 30
	p.Arg236His	AG	–	0.23 (0.008), *n* = 152	0.23 (0.01), *n* = 61
		GG	0.26 (0.01), *n* = 75	0.23 (0.008), *n* = 150	0.23 (0.01), *n* = 36
		P-value	p.Arg236His, >0.10	p.Asp298Asn, <0.05	Interaction, >0.10
ADGTEST		AA	–	–	0.64 (0.01), *n* = 30
	p.Arg236His	AG	–	0.68 (0.007), *n* = 152	0.67 (0.008), *n* = 61
		GG	0.71 (0.008), *n* = 75	0.69 (0.007), *n* = 150	0.71 (0.01), *n* = 36
		P-value	p.Arg236His, <0.01	p.Asp298Asn, <0.10	Interaction, <0.05

Average BF, average back fat thickness on three sites; Lumbar BF, Back fat at last lumbar; 10th rib BF, Back fat at the 10th rib; Last rib BF, Back fat at last rib; ADGW, average daily gain from birth to weaning; ADGTEST, average daily gain on test; LEA, Loin eye area.
[1]Only one animal with combined genotype AA(p.Arg236His)/AG(p.Asp298Asn) was observed. It was then excluded for association analyses.

fat. No significant interaction was observed between these two SNPs for fatness traits and ADGW, but a significant ($P < 0.05$) interaction was found for ADGTEST. For animals with genotype GG at p.Asp298Asn, the ADGTEST phenotype depended on the p.Arg236His allele present, with a GG/GG genotype showing the greatest growth rate.

Linkage mapping and QTL analysis

MC4R was mapped at around 67cM on SSC1 using the ISU B × Y population. The order of two non-synonymous SNPs on SSC1 was *S0331*-2.0cM-p.Arg236His-0.6cM-p.Arg298Asn-17.5cM-*Sw974*. Although the physical distance between these two SNPs was 185 bp, it is surprising that there was no strong LD relationship between p.Arg236His and p.Asp298Asn, but this may reflect that the origins of the SNPs and haplotypes.

In the previous QTL studies using the B × Y pig family, a QTL related to 10th rib back fat at the 5% genome-wide threshold was mapped around 30 cM on SSC1, and three QTL related to average back fat, lumbar back fat and last rib back fat at the experimental threshold were mapped around 73 cM. There was no QTL related to average daily gain on test in this family (Malek *et al.* 2001). *MC4R* was added to the QTL analysis as a marker using the haplotype. The peak shifted and increased in value for back fat (Table 4). As additional confirmation of the importance of *MC4R*, the genotypes of p.Arg236His, p.Asp298Asn and the *MC4R* haplotypes were included as individual fixed effects in the model, and the *F*-value peaks for fatness traits dropped where *MC4R* maps. The drop-in *F*-value was large when the haplotype was included (from 6.54 to 2.41) and it also decreased with the individual SNPs but less so than in the area where *MC4R* maps (Table 4). The QTL analysis assumes that genotypes segregated between the founder breeds, which are satisfied better with the p.Arg236His SNP than the p.Asp298Asn SNP, which segregated in both founder breeds.

Table 4 The distribution of *MC4R* genotypes/haplotypes in founder animals of the ISU Berkshire × Yorkshire pig resource family, and their influences on the mapping of QTL related to average back fat.

	QTL peak position (cM)	*F*-value[1]	Genotype/haplotype[2] distribution in the Berkshire founders			Genotype/haplotype distribution in the Yorkshire founders		
Microsatellites without *MC4R* effect	74	6.54		–			–	
With *MC4R* haplotype as marker effect	65	6.73	11 (0)	12 (2)	13 (0)	11 (4)	12 (1)	13 (3)
With *MC4R* p.Asp298Asn effect as fixed	73	6.22	AA (0)	AG (2)	GG (0)	AA (4)	AG (4)	GG (0)
With *MC4R* p.Arg236His effect as fixed	73	3.86	AA (0)	AG (0)	GG (2)	AA (0)	AG (3)	GG (5)
With *MC4R* haplotype effect as fixed	75	2.41	11 (0)	12 (2)	13 (0)	11 (4)	12 (1)	13 (3)

The number in the parentheses indicates the number of animals. SNP genotyping was unsuccessful for one Yorkshire founder.

[1]The chromosome-wise *F*-statistic threshold at the 5% level is 5.08, and the *F*-statistic thresholds at the 5% and 1% genome-wise level are 8.22 and 9.96 respectively (Malek *et al.* 2001).

[2]Only three haplotype combinations (genotypes) were observed in the founders. Haplotypes 1 and 2 were found at a frequency of 0.5 in the Berkshire founders while all three haplotypes were observed in Yorkshires (with frequencies of 0.75, 0.06 and 0.19 respectively).

Discussion

In humans, about 70 missense and several promoter variants have been identified in *MC4R* (Lubrano-Berthelier *et al.* 2003a,b; Tao 2005; Hinney *et al.* 2006; Valli-Jaakola *et al.* 2006). However, until now, only a few variants have been found in pigs, which may reflect the long-term artificial selection focus for reduced fatness and increased growth performance. Several mutants at position 236 have been found in humans, including p.Arg236His (as in pig), p.Arg236Gly and p.Arg236Trp. The p.Arg236His is a like-wild-type mutation, and describes mutants which usually result in a cAMP response similar to or slightly higher than that of wild-type (Hinney *et al.* 2006), whereas p.Arg236Gly could disrupt a dibasic cleavage site in pro-opiomelanocortin and lead to an inherited susceptibility to obesity (Challis *et al.* 2002). Although human polymorphisms have not been found at the other two polymorphic sites found in pigs (positions 59 and 298), polymorphisms at adjacent positions p.Ser58Cys, p.Pro299His and p.Pro299Ser have been found. According to the functional classification of *MC4R* mutations, p.Ser58Cys and p.Pro299His belong to intracellularly trapped mutants that suggest mutant receptors can be produced, but they are retained intracelluarly (Tao 2005). These results suggest that *MC4R* variants may have different receptor signal transduction properties that will cause partially or totally impaired functional effects depending on the specific change.

The association of p.Asp298Asn with back fat and growth rate traits has been confirmed in several studies with pigs derived from different backgrounds and it is considered useful for marker assisted selection (Kim *et al.* 2000a, 2004b; Hernández-Sánchez *et al.* 2003; Houston *et al.* 2004; Bruun *et al.* 2006; Jokubka *et al.* 2006; Meidtner *et al.* 2006; Óvilo *et al.* 2006; Van den Maagdenberg *et al.* 2007). However, this polymorphism does not have much impact on variation in fatness in the ISU resource family (Table S1 and Fig. 1a). Another non-synonymous mutation, p.Arg236His, which does not exhibit LD with p.Asp298Asn, shows significant associations with back fat and growth on test in this study. These findings suggest that variation in *MC4R* has an effect on growth rate and back fat traits, but that the effects are variable depending on the SNP and possibly interactions between these SNPs (Table 3). In this study, the SNP p.Asp298Asn influences growth rate over the pig's lifetime, while the SNP p.Arg236His appears to impact on growth rate after weaning (in this case when fed *ad libitum* at the fastest-growing stage). The p.Arg236His polymorphism (or the other SNPs in LD with it), if present, could be useful indicators for lean quality and belly thickness evaluation in commercial pigs because of their association with last rib back fat and LEA, which are two major characteristics for grades of pork carcasses (United States Standards for Grades of Pork Carcasses). In humans, the inconsistency of the associations of *MC4R* with obesity phenotypes in populations implied that multiple functional defects of *MC4R* related to different biological pathways, or that mutations occurring in regulatory regions rather than transcription regions are major causes for fatness development (Govaerts *et al.* 2005; Tao 2005; Chambers *et al.* 2008; Loos *et al.* 2008). This may also be the case here, although it may also depend on genetic background and/or interaction between the polymorphisms.

Three major haplotypes of *MC4R* gene were identified in the study, which were consistent with the findings of Meidtner *et al.* (2006) in a Mangalitsa × Piėtrain pig resource family. Unfortunately, the frequency of the p.Arg236His polymorphism was relatively low in that study so that animals of haplotype 3 were not frequent. To try to disentangle the effects of these SNPs, we carried out association analyses of haplotype combinations and copy number of each haplotype (0, 1 or 2 copies) in the population. Haplotype 2, defined as the wild-type haplotype by Meidtner *et al.* (2006), was associated with all of the back fat traits but not growth rate. Both haplotype 1 and haplotype 3 were associated with ADGTEST, average back fat and lumbar fat

traits. Animals homozygous for haplotype 3 are fatter (for average back fat and lumber back fat), and grew more slowly than the other two haplotypes. This is surprising as faster growing animals are normally fatter, as is seen with the p.Asp298Asn polymorphism (Kim *et al.* 2000a). The discrepancy might result from the relatively small size of the analysed population or other unknown reasons (Zhao *et al.* 2003). It can be seen that animals that have a G at codon 298 may have either of the alleles at codon 236. Thus, the frequency of the p.Arg236His polymorphism in a population may impact on the effect of the p.Asp298Asn polymorphism. To explore this hypothesis, we carried on the association analyses of haplotype combinations between these two important non-synonymous SNPs. There was no significant interaction between the two SNPs for the back fat traits and ADGW, but a significant interaction was found for ADGTEST (Table 3). Significant differences between p.Arg236His genotypes were found within the GG genotype of p.Asp298Asn, while there was no significant difference between p.Asp298Asn genotypes within the GG genotype of p.Arg236His. Meanwhile, based on the association results of haplotype combinations shown in Table 2, there was significant difference between 11 and 12 that can be accounted for by the GG genotype of p.Arg236His, and similarly, there were significant differences between 22 and 33, 23 and 33, which were related to the genotype of p.Arg298Asn. These results also suggest the existence of interaction between p.Arg236His and p.Asp298Asn on ADGTEST.

In addition, the QTL peaks around 73 cM on SSC1 were greatly reduced when the p.Asp298Asn and p.Arg236His genotypes and haplotypes were included as fixed effects in the model, indicating that they could absorb a significant proportion of the variation seen for fatness measures in this region, providing further support for *MC4R* as a candidate gene for the fat QTL. Similar results were found in a Landrace × Hampshire intercross population when the genotype of p.Arg298Asn was treated as fixed effect in the model (Bruun *et al.* 2006). These results suggest re-examination of *MC4R* in populations where the original mutation appeared would have no effect. Furthermore, both of the non-synonymous mutations are of significance for fatness traits, but the effect of p.Arg236His was greater than that of p.Asp298Asn in the B × Y resource family. It is not surprising since this newly identified mutation only originated in one founder breed, but p.Asp298Asn segregated within each breed (Table 4). However, there was no evidence for a growth rate QTL at this location in this population, even though there is an effect of *MC4R* genotype on growth.

In the pig, allele G (Asp298) of p.Asp298Asn was considered to be the ancestral allele, and individuals carrying this allele have less back fat thickness, slower growth rate and lower feed intake compared with those carrying Allele A (Asn298) (Kim *et al.* 2000a, 2004b). As for p.Arg236His, allele G representing 236Arg is well-conserved among different pig breeds, suborder Suiformes (pig like) and other mammalian species (Kim *et al.* 2004a; Stäubert *et al.* 2007), and here it was found to be associated with faster growth rate. Allele A, representing His236, has only been found in a few pig breeds including Vietnamese Potbelly and Piėtrain, as well as Large White (Yorkshire) used in this study (it is also highly conserved within fish species) (Kim *et al.* 2004a; Meidtner *et al.* 2006; Stäubert *et al.* 2007). This allele was associated with slower-growing and fatter animals. It could be speculated that haplotype 3 -CCTAGT-, which includes 236His and 298Asp, was derived from a different origin to the other haplotypes. Its presence in Yorkshire, as well as Vietnamese Potbelly, suggests that it may be of Asian origin. The 236His allele appears within a different haplotype and may be of Asian origin but still exists in some western breeds influenced by Asian breeds. It may also have been eliminated in most commercial breeds under long-term artificial selection for faster growth and leaner meat. In this sense, p.Asp298Asn may have more significance for selection programmes in western breeds. In practice, however, both non-synonymous SNPs can be utilized for marker-assisted selection in populations where p.Arg236His is segregating, particularly for populations where the association of p.Asp298Asn was small or non-significant in previous studies. For example, although a significant interaction with line type was not identified in the original study of p.Asp298Asn (Kim *et al.* 2000a), inspection of that data in the light of the results in this study indicates that the effect within the Large White line was not significant (although the number of animals in each individual line was relatively small). This suggests that the p.Arg236His polymorphism may have been segregating in this line and also in the Meishan/Large White line studied by Kim *et al.* (2000a). Further analysis of p.Arg236His is worthwhile to clarify the possible selection against this mutation of *MC4R* gene in pigs of different origins, and the interaction effects between this SNP and p.Asp298Asn.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Association results between six SNPs in *MC4R* and fatness and growth rate traits in the ISU Berkshire × Yorkshire pig resource family, respectively.

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