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A single nucleotide polymorphism in the porcine cathepsin K (*CTSK*) gene is associated with back fat thickness and production traits in Italian Duroc pigs

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Abstract Cathepsin K (*CTSK*) was selected as a candidate gene for fat deposition in pigs because recently, in human and mouse, it was shown that this lysosomal proteinase is an obesity marker. A single nucleotide polymorphism (SNP) was identified in intron 4 of the porcine *CTSK* gene (g.15G>A; FM209043). Allele frequencies of this polymorphism were analysed in seven pig breeds. Radiation hybrid mapping confirmed the localization of *CTSK* to porcine chromosome 4, close to the *FAT1* QTL region. Three populations of pigs (one Italian Large White and two Italian Duroc groups of pigs) were selected for association analysis. In the Italian Large White breed the g.15G>A SNP was not informative. Association analysis including all Italian Duroc pigs showed that the *CTSK* marker was associated with back fat thickness and lean cuts ($P < 0.01$), and average daily gain and feed:gain ratio ($P < 0.05$) estimated breeding values.

Keywords Association study · Back fat thickness · *CTSK* · Duroc pigs · SNP

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Introduction

Fat deposition in pigs has been the matter of a large number of studies showing that a candidate gene approach, combining in several cases QTL information, can be useful to identify DNA markers associated with fatness as well as other correlated traits [1–3].

Cathepsins are members of a large family of lysosomal proteinases that initially were recognized as non specific protein scavengers affecting cellular and tissue anabolic and catabolic processes [4, 5]. From subsequent studies, several cathepsins resulted to have cell, highly specific and directed proteolytic activities including antigen, hormone and proenzyme processing, with effects on immune response and biochemical pathway regulation and activation in different tissues [4, 5].

Cathepsin K (*CTSK*) was originally identified as an osteoclast-specific lysosomal cysteine proteinase involved in degradation of bone matrices such as type I collagen [6, 7]. Recently, it was shown that *CTSK* could affect the innate immune response to pathogen DNA by compromising TLR9 signaling [8]. In addition to this role, another specific function of *CTSK* has been shown in adipocyte differentiation. Increased expression of the gene was observed in adipose tissue of experimentally obese mice and in human with obese phenotype [9, 10]. Moreover, *CTSK* deficient mice displayed significantly lower body weight and lower level of fat deposition than wild type animals strengthening the role of this proteinase as a marker in human obesity [11]. The human *CTSK* gene maps on chromosome 1q21 (NCBI Human Genome Build 36.3; http://www.ncbi.nlm.nih.gov/projects/genome/guide/human/), a region that is syntenic with porcine chromosome 4 (SSC4) [12], where several QTL for fat deposition and production traits have been identified [13–16].

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According to these functions and data, *CTSK* can be considered a candidate gene for fatness and correlated traits in pigs as already shown for other cathepsin genes [17].

Here, we show that a novel single nucleotide polymorphism (SNP) in the porcine *CTSK* gene is associated with back fat thickness and other production traits in Italian Duroc pigs.

Materials and methods

Animals and traits

The structure of the sib-tested Italian Large White and Italian Duroc populations is based on triplets of pigs of the same litter (two females and one castrated male) that are performance tested and slaughtered at about 155 kg for the genetic evaluation of a candidate boar from the same litter. Among these populations, three groups of pigs were selected for association analysis with a marker we identified in the porcine *CTSK* gene. The first two groups of pigs were chosen using a selective genotyping approach and were: (1) 100 Italian Large White pigs with extreme estimated breeding value (EBV) for back fat thickness (BFT; 50 with the most negative and 50 with the most positive values; 70 females and 30 castrated males) selected among 3,591 tested animals of this breed slaughtered in a 4 year period (1996–2000); (2) 100 Italian Duroc pigs with extreme EBV for visible intermuscular fat (VIF; 50 with the most negative and 50 with the most positive values; 58 females and 42 castrated males) selected among 1,225 sib tested Italian pigs of this breed slaughtered in a 4 year period (1996–2000). BFT was recorded *post mortem* at the level of the *gluteus medius* muscle and is expressed in mm. VIF is a measure recorded as a categorical trait only in Italian Duroc pigs that resemble intermuscular fat percentage and is expressed in units of standard deviation of the probability to transmit intermuscular fat (ANAS, http://www.anas.it). EBV means ± SD for the extreme groups of Italian Large White and Italian Duroc pigs were −11.40 ± 1.07 mm and +10.69 ± 3.19 mm (BFT) and −2.35 ± 0.27 and +2.17 ± 0.34 (VIF), respectively.

The third group of pigs was made of 211 sib-tested Italian Duroc animals (138 females and 73 castrated males, obtained from 92 different sires) not selected by any phenotypic or genotypic criteria (slaughtered from 1995 to 2003). For these pigs, EBV were obtained for BFT, VIF, average daily gain (ADG; calculated from 30 to 155 kg of live weight with a *quasi* ad libitum nutritive level and expressed in grams), weight of lean cuts (LC; the sum of neck and loin weights and expressed in kilograms), and feed:gain ratio (FGR; obtained from feed intake recorded daily and body weight measured bimonthly). EBV for these traits were calculated as described below.

Other unrelated pigs of different breeds, for which phenotypic traits were not available, were used for allele frequency evaluation of the *CTSK* marker (Table 1).

Table 1 Allele frequencies of the *CTSK* g.15G>A polymorphism in different pig breeds

Breeds	No. of pigs	Allele frequency	
		g.15G	g.15A
Italian Large White	45	0.944	0.056
Italian Duroc[a]	311	0.897	0.103
Italian Landrace	20	0.925	0.075
Pietrain	12	1.000	0.000
Belgian Landrace	18	1.000	0.000
Hampshire	12	0.875	0.125
Meishan	12	0.708	0.292

[a] Allele frequencies are calculated using the 311 Italian Duroc pigs investigated for association analysis

Identification and analysis of polymorphism

BLASTN analysis of DNA databases (http://www.ncbi.nlm.nih.gov/Blast.cgi) with the cDNA sequence of the porcine *CTSK* gene (EMBL accession number AF292030) [18] was used to identify porcine genomic sequences containing this gene. The identified genomic sequence (EMBL accession number CU463875) was used to design PCR primers (primer pair 1: forward 5′-ctcaaaggcagacagcaatg-3′, reverse, 5′-tcattttgatccccaagtca-3′; primer pair 2: forward 5′-cttgggcgatatggtgagtt-3′, reverse 5′-aattctttgccctctctcctg-3′) that amplified two regions of 388 bp (including part of the 5′-flanking region, exon 1 and part of intron 1) and 359 bp (including intron 4, exon 5 and part of intron 5) of the porcine *CTSK* gene.

DNA was isolated from blood or meat or hair roots using standard protocols. PCR was carried out using a PT-100 (MJ Research, Watertown, MA, USA) thermal cycler in a final volume of 20 µl that included 10-100 ng of genomic DNA, 10 pmol of each primer, 2.0 mM $MgCl_2$, 2.5 mM each dNTP, 1 U EuroTaq (EuroClone Ltd., Paington, Devon, UK) DNA polymerase. The PCR profile was the following: an initial step of denaturation for 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 72° C; the final extension step was for 5 min at 72°C. Sequencing of the amplified fragments obtained in a panel of 11 different pigs (two Italian Large White, three Italian Duroc, two Italian Landrace, one Belgian Landrace, one Hampshire and two Meishan) was obtained using the same PCR primers and the Big Dye v3.1 kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions

were loaded on an ABI3100 Avant sequencer (Applied Biosystems) and electropherograms were visualized and aligned with CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA). An identified single nucleotide polymorphism was analysed by PCR-RFLP. Five µl of PCR product was digested overnight at 37°C with 3 U of restriction enzyme *Fsp*BI (MBI Fermentas, Vilnius, Lithuania) in a final volume of 25 µl containing 1× enzyme reaction buffer. PCR-RFLP products were resolved on 10% polyacrylamide/bis-acrylamide 29:1 gels stained with ethidium bromide. Allele g.15G resulted in a fragment of 361 bp whereas allele g.15A originated two fragments of 328 and 33 bp.

Radiation hybrid (RH) mapping

The INRA-Minnesota 7000 rads RH panel (IMpRH panel) [19] consisting of 118 rodent-porcine hybrid cell lines was screened by means of PCR using primer pair 2. No PCR fragment was obtained from the control rodent genomic DNA. The PCR reactions were visualized on 10% polyacrylamide/bis-acrylamide 29:1 or 2% agarose gels. The results of RH PCR products were analysed with the IMpRH mapping tool accessible through the http://imprh.toulouse.inra.fr/ web address [20].

Statistical analyses

Estimated breeding value were calculated using a BLUP-multiple trait-animal model [17]. Models were different for each trait and included fixed effects of batch in test, sex, age at the beginning of test, age of sow, weight at slaughter, age at slaughter and inbreeding coefficient, and the random effects of litter, individual permanent environment and animal. For the first two groups of pigs chosen for a selective genotyping approach, Fisher's exact test of significance (two-tailed) of differences of allele frequency between the positive and negative groups was tested. The EBV distribution for the BFT, ADG, LC and FGR traits in the second group of pigs (Italian Duroc animals), chosen according to the extreme values for VIF EBV, did not differ from the normal distribution (Shapiro–Wilk test for these traits was $P > 0.15$). Thus, this group of Italian Duroc pigs was merged with the third group of animals of the same breed (a total of 311 Italian Duroc pigs) and used to test the association between the genotypes at the *CTSK* gene and the calculated EBVs for BFT, ADG, LC and FGR using the general linear model (GLM) procedure of SAS, release 8.02 (SAS Institute Inc., Cary, NC). For VIF EBV, the same association analysis was obtained using only 211 Italian Duroc pigs of the third group. The model included only the fixed effects of the genotypes as all other factors contributing to the variability of the investigated traits were already considered in the calculation of their EBV. Following the reasoning already discussed by Russo et al. [17], in this study that included only five marker trait tests, a *P* nominal value of 0.05 was considered as a threshold to declare significance.

Results and discussion

From the resequencing of two targeted regions of the porcine *CTSK* gene (a whole of 8,239 sequenced bp considering the 11 analysed animals) we identified a SNP in intron 4 (g.15G>A; EMBL accession numbers for the two alleles are FM209043 and FM209044, respectively), according to the human *CTSK* exon-intron gene organization. This polymorphic site is different from the DNA marker recently reported in this gene by Ramos et al. [21]. Of the sequenced pigs, one Meishan had genotype g.15AA and two Duroc had genotype g.15GA whereas the genotype of the other animals was g.15GG. The low frequency of allele g.15A was confirmed in a larger sample of pigs of different breeds for which this polymorphic site was analysed by PCR-RFLP (Table 1).

Recently, Hamasima et al. [22] reported the RH mapping of the porcine *CTSK* gene to chromosome 4, confirming comparative mapping information with human chromosome 1 where this gene is located. However, looking at the details of the reported mapping information, it turned out that the porcine expressed sequence tag (GenBank accession number DN111520) used for primer design did not correspond to *CTSK* but was a portion of the proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (*PSMD4*) gene. Therefore, we wanted to confirm the mapping position of the porcine *CTSK* gene and the identified SNP by RH mapping. Analysing the IMpRH panel the retention frequency of the amplified *CTSK* gene fragment was 31% and the closest marker obtained by two-point analysis was *Sw512* (distance = 39 cR; LOD = 10.72), already placed on the RH map of SSC4 [23]. This data confirmed its mapping position deduced from comparative data and the information obtained by BLAST analysis in the Pre-Ensembl *Sus scrofa* database (http://pre.ensembl.org/Sus_scrofa/index.html) (data not shown). According to Berg et al. [24], this mapping position might exclude *CTSK* from the most likely chromosome region of the *FAT1* QTL that should be located just a few cR towards the centromeric region. However, QTL patterns of this chromosome are quite complex. More QTL (with probably more than two alleles at the same QTL), covering the *CTSK* map position, seem to affect fat deposition, growth and several other production traits in different pig populations [16, 25]. For this reason, *CTSK* still remains a positional and functional candidate gene.

Association studies with the g.15G>A SNP were carried out using different approaches and populations: (i) a selective genotyping approach for BFT in Italian Large White pigs; (ii) a selective genotyping approach for VIF in Italian Duroc pigs; (iii) an association study in a random Italian Duroc sib-tested population.

In the Italian Large White pigs used in the selective genotyping approach, we were not able to test any allele frequency difference between the two extreme groups because only one animal (of the positive group) had genotype g.15GA and all others had genotype g.15GG (two tailed Fisher's exact test, $P \approx 1$). These results confirmed the low frequency of allele g.15A in this breed as suggested by the allele frequency data reported in Table 1 for another group of Italian Large White pigs. Thus, this marker seems poorly informative in this breed and other polymorphisms should be identified in this gene for association studies in Italian Large White animals.

The selective genotyping approach for VIF EBV in the Italian Duroc sib-tested pigs did not show any allele frequency difference from the two extreme groups. Indeed, both groups had exactly the same allele frequency (g.15G = 0.870), thus it seems that this marker is not associated with VIF.

However, as this polymorphism segregates in Italian Duroc pigs, we studied its association with several other EBV in a larger population considered as a random sample of pigs. Only two animals had genotype g.15AA, therefore this genotypic class was merged with the second less frequent genotypic class, the heterozygous animals (g.15GA). Results of the association analysis are reported in Table 2. Overlapping results were obtained excluding from the association tests the animals with the less frequent genotype (data not shown). No association was observed for VIF confirming the results of the selective genotyping approach. However, *CTSK* genotypes were significantly associated with BFT and LC ($P < 0.01$) and ADG and FGR ($P < 0.05$). The most frequent genotype (g.15GG) had lower BFT, higher LC, higher ADG and lower (favourable) FGR. The effects of the *CTSK* marker was in same direction as expected from the correlations between these traits. The higher frequency of the g.15GG genotype in this breed (and may be in the other breeds) might be due to selective pressure towards meat production efficiency. A higher frequency of allele g.15A was observed in the Meishan pigs (Table 1), that are notoriously less efficient in lean meat deposition.

Several studies on genes mapping around the *FAT1* region showed striking peculiarities on intra- and inter-breed nucleotide variation, diversity in haploblock structure and different effects on production traits [25–28] suggesting the action of more genetic processes (balancing selection and directional selection, admixture, bottleneck events) in shaping the genetic structure of different SSC4 chromosome regions in different pig lines or breeds. Even if more studies are needed to characterize the porcine *CTSK* gene and to evaluate if different genetic processes operated in shaping its variability, our results indicated that the analysed marker might be in close linkage disequilibrium with (a) functional mutation(s) in this gene or in (a) close gene(s). For the role that *CTSK* plays on adipogenesis [9–11], we could speculate a possible effect of this gene on fat deposition in Duroc pigs.

In a candidate gene approach, we recently showed that a few other cathepsin genes (cathepsin D, cathepsin F, cathepsin H and cathepsin Z) are associated with back fat thickness and other production traits in Italian Large White pigs [17]. To some extent, together with reports on the involvement of other proteinases on obesity development [29], these results on porcine cathepsin genes, if confirmed by functional studies, might provide additional clues on the key roles of proteolytic mechanisms in adipogenesis and fat mass development.

Table 2 Association analysis between *CTSK* marker and EBV in the Italian Duroc pigs

EBV	Genotypes[a]		*P*
	g.15GG (n = 249)	g.15GA + g.15AA (n = 62 + 2)	
ADG	+30.919 (1.889)	+21.594 (3.711)	0.026
BFT	−2.060 (0.254)	−0.556 (0.499)	0.007
FGR	−0.165 (0.010)	−0.113 (0.020)	0.020
LC	+2.069 (0.130)	+1.208 (0.256)	0.003
	g.15GG (n = 173)	g.15GA + g.15AA (n = 36 + 2)	
VIF	−0.391 (0.082)	−0.439 (0.176)	0.806

Least square means are reported with their standard errors (in parenthesis)

EBV estimated breeding value, *ADG* average daily gain, *BFT* back fat thickness, *FGR* feed:gain ratio, *LC* lean cuts, *VIF* visible intermuscular fat

[a] The less frequent genotypic class (g.15AA, two animals) was merged with the heterozygous class (g.15GA) for a total of 62 pigs considering the group of 311 pigs (association analysis with ADG, BFT, FGR and LC) or for a total of 38 animals considering the group of 211 pigs (association analysis with VIF). The number of g.15GG pigs was 249 and 173 considering the 311 and the 211 Italian Duroc pig groups, respectively

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