Livestock Science 190 (2016) 27–30

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Short communication

An association analysis between a missense polymorphism at the pig *PCSK9* gene and serum lipid and meat quality traits in Duroc pigs

Wojciech Chański [a,1], Rayner González-Prendes [a], Anna Castelló [a,b], Jordi Jordana [b], Arianna Manunza [a], Raquel Quintanilla [c], Marcel Amills [a,*]

[a] Department of Animal Genetics, Center for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB), Campus Universitat Autònoma de Barcelona, Bellaterra 08193, Spain
[b] Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Campus Universitat Autònoma de Barcelona, Bellaterra 08193, Spain
[c] Animal Breeding and Genetics Program, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Torre Marimon, Caldes de Montbui 08140, Spain

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form
23 May 2016
Accepted 24 May 2016

Keywords:
Proprotein convertase subtilisin-like kexin type 9
Palmitelaidic
Swine

ABSTRACT

A genome-wide association analysis in a Duroc pig population allowed us detecting a genomic region on pig chromosome 6 (141–147 Mb) that was associated with serum cholesterol (CHOL), triglyceride (TRIG) and low-density lipoprotein (LDL) concentrations. This region contains the proprotein convertase subtilisin-like kexin type 9 (*PCSK9*) gene (SSC6, 145 Mb), which has a key role in the regulation of CD36, LDL receptor and very low density lipoprotein (VLDL) receptor levels. In the current work, we have genotyped by pyrosequencing a missense *PCSK9* c.1222G > A mutation (E408K) in 273 Duroc pigs. The performance of an association analysis with the GEMMA software did not reveal any association between *PCSK9* genotype and serum lipid concentrations, evidencing that this polymorphism is not the causal mutation of the CHOL, TRIG, and LDL SSC6 QTL. However, we detected an association, that was highly significant at the nominal level, between *PCSK9* genotype and palmitelaidic content at the *gluteus medius* muscle (*P*-value = 0.008). There is evidence that PCSK9 induces the degradation of CD36, a key long-chain fatty acid transporter, and that it may decrease the uptake of palmitate. However, the E408K polymorphism analysed in the current work is not predicted to be deleterious, suggesting that the associations found are probably due to the linkage of this polymorphism with a causal mutation yet to be found.

1. Introduction

In a previous genome-wide association analysis for serum traits in a Duroc pig population (Lipgen population), we detected the existence of a genomic region on SSC6 (~141–147 Mb) that displays significant associations with cholesterol (CHOL) and low density lipoprotein (LDL) concentrations at 190 days-of-age as well as with triglyceride (TRIG) levels at 45 and 190 days-of-age (Manunza et al., 2014). Interestingly, this genomic region contains the proprotein convertase subtilisin-like kexin type 9 (*PCSK9*) gene (SSC6, 145 Mb), that encodes a serine-protease that mediates the degradation of the LDL receptor bound to the plasma membrane of hepatocytes (Roubtsova et al., 2011). There is evidence that gain-of-function mutations in PCSK9 produce dominant hypercholesterolemia in humans (Abifadel et al., 2003), while recessive loss-of-function mutations diminish significantly the risk of coronary heart disease (Cohen et al., 2006). Moreover, Roubtsova et al. (2011) showed that *PCSK9* knockout mice have an augmented rate of *in vivo* fatty acids uptake in adipose tissue and hypothesized that such outcome might be due to the upregulation of the very low density lipoprotein (VLDL) receptor. Given that *PCSK9* is a positional and functional candidate gene for the CHOL, TRIG, and LDL QTL detected on SSC6 by Manunza et al. (2014), we aimed to investigate the association of its polymorphism with serum lipid concentrations as well as a wide array of meat quality traits recorded in the Lipgen population.

2. Materials and methods

2.1. Animal material and phenotype recording

As animal material, we have used a Duroc pig population (Lipgen population) previously described by Manunza et al. (2014). Phenotypic records were obtained in 273 castrated male pigs

* Corresponding author.
E-mail address: marcel.amills@uab.cat (M. Amills).
[1] Current address: University of Copenhagen, Faculty of Health and Medical Sciences, Department of Veterinary Clinical and Animal Sciences, Section of Animal Genetics, Bioinformatics & Breeding, Grønnegårdsvej 3, 1870 Frederiksberg C, Denmark.

http://dx.doi.org/10.1016/j.livsci.2016.05.011
1871-1413/

belonging to 5 half-sib families. Pigs were bred in the experimental station at the Centre de Control Porcí (CCP) of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) and distributed in 4 batches managed under standard intensive conditions. Barrows were fed *ad libitum* a diet with a net energy concentration of 2450 kcal/kg until they reached a live weight of 90 kg (around 150 days of age) Afterwards, diet was readjusted to a net energy concentration of 2375 kcal/kg. Development and management conditions of this population have been thoroughly described by Gallardo et al. (2008). Regarding animal care, all experimental management and sampling procedures employed in the current study followed the ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

Measurements of serum CHOL, TRIG, LDL and high density lipoprotein (HDL) concentrations at 45 and 190 days after birth (Gallardo et al., 2008) and intramuscular fat (IMF) content and composition in the *gluteus medius* (GM) and *longissimus dorsi* (LD) muscles (Vidal et al., 2005; Gallardo et al., 2009; Gallardo et al., 2012) were performed as previously reported. Electric conductivity (EC), ultimate pH (pH24) and colour measurements were determined 24 h postmortem following the protocols reported by Gallardo et al. (2012). Meat lightness (L*), redness (a*), and yellowness (b*) were recorded in duplicate with a Minolta spectrophotometer (Gallardo et al., 2012). Cholesterol content was determined using a high-performance liquid chromatography approach (Cayuela et al., 2003).

2.2. Characterization of the PCSK9 polymorphism in the Lipgen population

Genomic DNA was isolated from blood samples following a protocol described by Vidal et al. (2005). The *PCSK9* single nucleotide polymorphism (SNP) analysed in the current work (c.1222G > A), which involves an amino acid substitution (E408K), was discovered in an experiment undertaking the whole-genome sequencing of five Duroc boars at a 30 fold-coverage (our unpublished data). This polymorphism is not novel since it had been previously reported in the Ensembl database (rs323706293, http://www.ensembl.org), Sequences flanking this SNP were used as templates to design primers with the PyroMark Assay Design software (Qiagen, Barcelona, Spain). A confirmatory test for primer specificity was performed with the Primer-Blast tool (www.ncbi.nlm.nih.gov/ tools/primer-blast).

The 146 bp targeted region was amplified with primers 5′-GGG AAC CAG GCC TCA TTG AT-3′ and 5′-GGC CAC CAC CAT CTC TCA CT-3′ (biotinylated primer). Polymerase chain reaction (PCR) contained 2.5 mM $MgCl_2$, 250 μM dNTPs, 0.3 μM of each primer, 1 U of AmpliTaq Gold DNA polymerase (Life Technologies, Madrid, Spain) and ~50–60 ng genomic DNA in a final volume of 25 μl. The thermal profile comprised one denaturation step at 95 °C for 10 min, and 35 cycles of 95 °C for 1 min, 64 °C for 1 min and 72 °C for 1 min Finally, a single extension step at 72 °C for 7 min was carried out. Amplification reactions were performed in a Veriti 96-Well Thermal Cycler (Life Technologies) and they were checked in 1.8% agarose gels.

Genotyping was performed by implementing a pyrosequencing protocol (King and Scott-Horton, 2007). The sequencing primer was 5′-CCA GGG TGA GCT CCG-3′. In order to immobilize the biotinylated DNA strand, 60 μl of a mix containing 2 μl of Streptavidin Sepharose High Performance beads of 34 μm (GE Healthcare, Barcelona, Spain), 38 μl of PyroMark Binding Buffer (Qiagen Iberia, Barcelona, Spain) and 20 μl of MilliQ water were added to 20 μl of PCR product. The biotinylated DNA strand, captured with a PyroMark Q96 Vacuum Workstation (Qiagen Iberia), was used as a template in pyrosequencing reactions containing 12 μl of sequencing primer at 0.3 μM diluted with PyroMark Annealing Buffer and PyroMark Gold Q96 reagents (Qiagen Iberia). Reactions were performed in a PSQ HS96 equipment and the quality of the pyrograms was evaluated with the PSQ HS96A 1.2 software (Qiagen Iberia).

In silico predictions about the functional consequences of amino acid substitutions were performed with the SIFT (Ng and Henikoff, 2003; http://sift.jcvi.org) and Panther cSNP (Mi et al., 2016; http://www.pantherdb.org/tools/csnpScoreForm.jsp) softwares. SIFT predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids, and it provides a score that can go from 0 to 0.05 (deleterious mutation) or 0.06–1 (tolerated substitution). In contrast, Panther cSNP uses sets of evolutionarily related sequences to estimate the probability of a given amino acid at a particular position in a protein, and it yields a probability of deleteriousness (*i.e.* probability that the variant under analysis will cause a deleterious effect on protein function).

2.3. Association analysis

The association analysis was performed for 271 successfully genotyped individuals. We took into consideration a wide array of meat quality and serum lipid traits (Supplementary Table 1) described in previous publications (Gallardo et al., 2008; Quintanilla et al., 2011; Gallardo et al., 2012). Statistical analyses were conducted with the GEMMA software (Zhou and Stephens, 2012). This statistical package assumes the following general model:

$$y_{ijk} = \mathbf{W\alpha} + \mathbf{X\beta} + Zu + \boldsymbol{\varepsilon}$$

$$u \approx MVN_m\left(0, \lambda\tau^{-1}K\right)$$

$$\varepsilon \approx MVN_n\left(0, \lambda\tau^{-1}I_n\right)$$

where $\boldsymbol{n}$ is the number of individuals, $\boldsymbol{m}$ is the number of groups, $\mathbf{y}$ is an $n \times 1$ vector of phenotypes, $\mathbf{W} = (\mathbf{w}_1, \mathbf{w}_2 \ldots \mathbf{w}_c)$ is an $n \times c$ matrix of covariates (fixed effects) including a column vector of 1, $\boldsymbol{\alpha}$ is a $c \times 1$ vector of corresponding coefficients including the intercept, $\mathbf{x}$ is an $n \times 1$ vector of marker genotypes, $\boldsymbol{\beta}$ is the effect size of the marker, $\mathbf{Z}$ is an $n \times m$ loading matrix, $\mathbf{u}$ is an $m \times 1$ vector of random effects, $\boldsymbol{\varepsilon}$ is an $n \times 1$ vector of errors, $\boldsymbol{\tau}^{-1}$ is the variance of the residual errors, $\boldsymbol{\lambda}$ is the ratio between the two variance components, $\mathbf{K}$ is a known $m \times m$ relatedness matrix, $\mathbf{I}_n$ is an $n \times n$ identity matrix and **MVN** denotes the n-dimensional multivariate normal distribution (Zhou and Stephens, 2012).

In our specific analysis, we assumed the following linear mixed models:

(1) For serum lipid concentrations

$$\boldsymbol{y}_{ijk} = \boldsymbol{\mu} + \boldsymbol{batch}_i + \boldsymbol{\beta_1 t}_j + \boldsymbol{\beta_2 g}_k + \boldsymbol{e}_{ijk} \quad \text{for records at 190 days of age;}$$

$$\boldsymbol{y}_{ijk} = \boldsymbol{\mu} + \boldsymbol{batch}_i + \boldsymbol{farm}_j + \boldsymbol{\beta_2} g_k + \boldsymbol{e}_{ijk} \quad \text{for records at 45 days of age;}$$

(2) For IMF content and composition and meat quality traits

$$\boldsymbol{y}_{ijk} = \boldsymbol{\mu} + \boldsymbol{batch}_i + \boldsymbol{\beta_1 t}_j + \boldsymbol{\beta_2 g}_k + \boldsymbol{e}_{ijk}$$

where $\boldsymbol{y}_{ijk}$ is the phenotypic record collected from the *i*th individual; $\boldsymbol{\mu}$ is the mean of the trait in the population; **batch** and **farm** are the systematic effects, batch of fattening (with 4 categories) and farm of origin (with 3 categories); $\boldsymbol{t_j}$ is a covariate that depends on the trait (Gallardo et al., 2008, 2012; Quintanilla et al., 2011): (1) live weight at slaughter for CHOL, HDL and LDL, (2) age at slaughter for TRIG, (3) IMF content in GM (for fatty acid traits measured in the GM muscle), (4) IMF content in LD (for fatty acid traits measured in the LD muscle), (5) backfat thickness (for IMF content measured in GM and LD), (6) weight at slaughter for meat quality traits measured in GM and LD; $\mathbf{g_k}$ is the vector of SNP