Differences in MC4R mRNA levels between Casertana and Large White pig breeds

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RIASSUNTO – Differenze tra i livelli di mRNA del gene MC4R nelle razze suine Casertana e Large White. Allo scopo di analizzare differenze nei livelli di espressione genica tra due razze suine a fenotipo estremo è stata costruita una popolazione sperimentale composta da 17 soggetti di razza Casertana e 16 di razza Large White. Impiegando l’approccio dei geni candidati è stato scelto e studiato il gene "Recettore-4 della Melanocortina", importante nella via metabolica che regola omeostasi energetica ed appetito. L’analisi è stata condotta utilizzando la tecnica della Real-Time PCR impiegando il gene della "Gliceraldeide 3-Postato Deidrogenasi" come riferimento. L’RNA totale è stato isolato da tessuto muscolare campionato al macello. Le analisi hanno evidenziato una grande variabilità individuale nell’espressione relativa del gene in ambo le razze (es.: valori di espressione compresi in un range di 0,05÷11,08 in Casertana e di 0,44÷4,86 in Large White). Inoltre è stata evidenziata una differenza significativa di espressione tra i campioni delle due razze considerate, risultato che però va verificato su un numero maggiore di campioni.

KEY WORDS: Casertana, MC4R, expression analysis.

INTRODUCTION – In the last few years the local or autochthonous breeds became more important for the typical products. The autochthonous breeds often possess valuable traits such as unique product qualities, disease resistance, adaptation to harsh conditions or poor quality feed. The Casertana pig, that is the subject of this study, is a local breed from the South of Italy characterized by slow growth and a massive accumulation of backfat. The Casertana breeding has been dismissed mainly for economic reasons, i.e. because of its long time to reach its mature body weight and preference of consumers for lean meat. The aim of this study was the analysis of Melanocortin-4 receptor (MC4R) expression variation in two groups of Casertana and Large White pigs. The MC4R has a key role in concert with leptin in regulating feed intake and energy balance (Kim et al., 2000; Huston et al., 2004). Loss of function mutations in the MC4R gene result in obesity (Vaisse et al., 1998; Matteri, 2001). A missense mutation in the porcine MC4R gene has been reported to be associated with growth, fatness and feed intake traits (Kim et al., 2000); although no effects have been detected in other populations (Park et al., 2002).

MATERIAL AND METHODS – Experimental population. A total of 33 animals were analysed, including 17 Casertana and 16 Large White. The animals balanced by sex and litter, of same age, were bred “en plain air” in the same environmental conditions to detect only the genetic differences. The muscle tissue was collected at slaughter when the animals reached the age of 11 months.

RNA extraction. For the RNA isolation, muscle tissue samples were collected post-mortem at slaughter, snap frozen in liquid nitrogen and stored at -80°C. RNA (total) was isolated using the Trizol Reagent Method (Life Technologies GIBCOBRL).

Real Time PCR Methods. Starting from the total RNA previous isolated the Reverse transcription was performed using the “TaqMan Reverse Transcription Reagents” kit (Applied Biosystems) in 100 µl volumes with 200 ng of total RNA, 1X TaqMan buffer, 5.5 mM MgCl₂, 500 µM dNTP, 2.5 µM Random Hexamer, 0.4 U/µl RNase Inhibitor, 1.5 U/µl Multiscribe RT. The thermal cycling conditions were: 25°C for 10 min, 48°C for 30
min, 95°C for 5 min. The PCR was conducted in 25 µl total volume using 1X TaqMan Buffer, 5.5 mM MgCl₂, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 100 nM probe, 200 nM of each primer, 0.01 U/µl AmpErase UNG, 0.025 U/µl AmpliTaq Gold DNA Polymerase and 5 µl cDNA. As a reference gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used. The primers and probes were designed with the “Primer Express 3.0” software from the following sequences (EMBL: AB21664, AF087937):

**MC4R:**
- Probe → PrMC4R 5‘ TGC CCG TCC TCC CAG GCA CT 3’
- Primer Forward → PFM4R 5‘ TCC TCA TGG CCA GAC TCC AC 3’
- Primer Reverse → PRMC4R 5‘ CCT TCA TGT TGG CAC CTT GG 3’

**GAPDH:**
- Probe → PrG3PDH 5‘ AGC ATC TCC TGA TTT CCA GTT TCC ATC CC 3’
- Primer Forward → PFG3PDH 5‘ AAC TCG ATC CCC CAA CAC AC 3’
- Primer Reverse → PRG3PDH 5‘ CCT AAG CCC CTC CTC TTC TT 3’

Table 1. Relative MC4R expression obtained using a common calibrator between the plates.

| Breed  | Mark | MC4R – Rel to 10CS | Expression range** |
|--------|------|-------------------|-------------------|
| Casertana | 1CS | 0.12 | 0.09 - 0.15 |
| Casertana | 14CS | 0.43 | 0.30 - 0.63 |
| Casertana | 13CS | 0.20 | 0.14 - 0.29 |
| Casertana | 19CS | 0.81 | 0.62 - 1.06 |
| Casertana | 20CS | 0.20 | 0.16 - 0.26 |
| Casertana | 12CS | 0.98 | 0.75 - 1.29 |
| Casertana | 18CS | 11.08 | 8.32 - 14.75 |
| Casertana | 9CS | 2.51 | 1.93 - 3.27 |
| Casertana | 7CS | 1.40 | 1.12 - 1.76 |
| Casertana | 15CS | 0.90 | 0.71 - 1.13 |
| Casertana | 17CS | 0.25 | 0.18 - 0.35 |
| Casertana | 3CS | 0.80 | 0.67 - 0.96 |
| Casertana | 6CS | 2.14 | 1.82 - 2.50 |
| Casertana | 16CS | 0.05 | 0.04 - 0.08 |
| Casertana | 11CS | 1.41 | 1.10 - 1.81 |
| Casertana | 5CS | 0.40 | 0.33 - 0.49 |
| Casertana | 10CS | 1.00 | 0.18 - 5.45 |
| **Group average** | **1.45 (0.85**)** |

*Group average calculated without considering the 18CS expression value.

**The range given for the MC4R relative expression is determined by the formula 2^ΔΔCt with ΔΔCt+s and ΔΔCt-s, where s is the standard deviation of the DDCt value.

min, 95°C for 5 min. The PCR was conducted in 25 µl total volume using 1X TaqMan Buffer, 5.5 mM MgCl₂, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 100 nM probe, 200 nM of each primer, 0.01 U/µl AmpErase UNG, 0.025 U/µl AmpliTaq Gold DNA Polymerase and 5 µl cDNA. As a reference gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used. The primers and probes were designed with the “Primer Express 3.0” software from the following sequences (EMBL: AB21664, AF087937):
The thermal cycling conditions used were: 50°C for 2 min, 95°C for 10 min, and 40 cycles a 95°C for 15 s and 60°C for 1 min using an “ABI PRISM 7700 Quantitative Real-Time PCR” system (Applied Biosystems). The amplification was conducted amplifying in singleplex the target and the reference genes using four replicates for each sample. The PCRs were performed using in each plate a common sample as calibrator (10CS). In addition all the PCRs were carried out twice in order to achieve a certain and reproducible result. The raw data were analysed using the “Comparative Ct or ∆∆Ct method” since the efficiency of the target and reference amplifications were checked and resulted approximately equal (absolute value of the slope <0.1) (User Bulletin ABI PRISM, 1997; Vu et al., 2000; Livak et al., 2001; Plaffl et al., 2002; Muller et al., 2002).

RESULTS AND CONCLUSION – It has been studied the Melanocortin-4 receptor differential expression between Casertana and Large White pig breeds. The analyses of each single plate results were done using as a calibrator the sample showing the lowest level of expression, according to the ∆∆Ct method (data not shown).

Moreover in order to compare the results obtained between the different plates, the ∆∆Ct method was performed using the 10CS sample as a common calibrator. In fact the mean of the Ct results obtained from the 10CS samples run in the different plates was used in the formula ∆∆Ct as the subtrahend. The relative expression values obtained analysing the plates one by one were not statistically significant compared with those obtained using the 10CS sample.

In addition the t-test (William Sealey Gosset, 1876-1937) performed indicated no statistically significant differences between the replicates of the amplifications.

The results presented in the table 1 are related to the data obtained using the 10CS common sample as calibrator in the analysis. As it is shown in the table, the intra-group differences were relevant in both breeds (variance in the Casertana breed: 6.64; variance in the Large White breed: 3.12). Instead the difference between the two genetic groups is not statistically supported. As it is shown in the table, the sample 18CS has the highest expression value. Considering this sample as an outgroup and rebuilding the analysis without it, the differences between the Casertana and the Large White breeds become statistically significant (p = 0.05).

The MC4R expression analysis has been a preliminary study that has shown important differences between the samples under investigation and the high individual differences found could be due to mutation in the MC4R gene or related sequences or to epistatic effects. Further investigations have to be done in order to compare the structural MC4R variations and the phenotypic changes in the same group of animals. Moreover additional analysis to investigate the remarkable expression of the 18CS will be completed.

REFERENCES – Applied Biosystems, 1997. User Bulletin #2 ABI PRISM 7700 Sequence Detection System. Huston, R.D., Cameron, N.D., Rance, K.A., 2004. A melanocortin-4 receptor (MC4R) polymorphism is associated with performance traits in divergently selected large white pig populations. Anim. Genet. 35: 386-390. Kim, K.S., Larsen, N., Rothschild, M.F., 2000. Rapid communication: linkage and physical mapping of porcine melanocortin-4 receptor (MC4R) gene. J. Anim. Sci. 78: 791-792. Kim, K.S., Larsen, N., Short, T., Plastow, G., Rothschild, M.F., 2000. A missense variant of porcine melanocortin-4 receptor (MC4R) gene is associated with fatness, growth, and feed intake traits. Mamm. Genome. 11: 131-135. Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−∆∆Ct Method. Methods 25: 402-408. Matteri, R.L., 2001. Overview of central targets for appetite regulation. J. Anim. Sci. 79(E. Suppl.): E148-E158. Muller, P.Y., Janovyak, H., Miserez, A.R., Dobbie, Z., 2002. Processing of Gene Expression Data Generated by Quantitative Real-Time RT-PCR. BioTechniques. 32: 1372-1379. Park, H.B., Carlborg, O., Marklund, S., Andersson, L., 2002. Melanocortin-4 receptor (MC4R) genotypes have no major effect on fatness in a Large-White X Wild Bear intercross. Anim. Genet. 33: 155-157. Plaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST®) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30(9): 1-10. Vaisse, C., Clement, K., Guy-Grand, B., Froguel, P., 1998. A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nature Genet. 20: 113-4. Vu, H.L., Troubetzkoj, S., Nguyen, H.H., Russeel, M.W., Mestecky, J., 2000. A method for quantification of absolute amounts of nucleic acid by (RT)_PCR and a new mathematical model for data analysis. Nucleic Acids Res. 28(7): 1-9. 2000.