Association analysis between single nucleotide polymorphisms in the promoter region of LEP, MYF6, MYOD1, OPN, SCD genes and carcass traits in heavy pigs

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Abstract

The purpose of the study was to evaluate associations between average daily gain (ADG) and DNA variability in the regulatory region of candidate genes in Italian Large White, Italian Duroc and Italian Landrace breeds for heavy pig production. The ADG of pigs was available as Estimated Breeding Values (EBVs) and Random Residuals (RRs). Within each breed, 200 individuals were sampled, 100 in the higher and 100 in the lower 5% tail of the normal distribution curve according to the EBVs for ADG of the populations (600 pigs overall). Six SNPs in promoter regions of 5 candidate genes (MYF6, MYOD1, OPN, SCD and two for LEP) were analysed. Allele frequencies were calculated and the SNPs for LEP, MYF6 and MYOD1 segregated in the 3 breeds, while polymorphisms in the OPN and SCD genes did not segregate in Italian Large White and Italian Landrace, and Italian Landrace respectively. Fisher’s exact test (two-tailed) for the distribution of allele frequencies in minus and plus variant groups was significant for LEP_1 and MYOD1 in Italian Large White, for LEP_1, LEP_2, MYOD1, OPN and SCD in Italian Duroc and for LEP_1, LEP_2 and MYOD1 in Italian Landrace. Several putative binding sites were detected in the promoter regions using MATINSPECTOR software. Analysed mutations may affect putative core sequences of binding sites for transcription factors involved in muscle and fat tissues deposition. However, variations in binding affinities of the sequences related to the SNPs and the expected differential gene expression require to be evaluated.

Introduction

Polymorphisms in the promoter region can modulate the amount of gene products without affecting the properties of the encoded protein (Miller et al., 1996; Mason et al., 1998). However, only with recent available release of the high-coverage Sscrofa10.2 assembly for chromosomes 1 to 18 and X of the pig genome (Groenen et al., 2012) a systematic study of the regulatory regions of the genes in pig is feasible, enabling to investigate association among DNA variability and phenotype. Among the phenotypic traits of animals for meat production, average daily gain, back fat thickness and feed conversion rate are the main indicators for the economic efficiency of the pig industry.

There is a large body of evidence reporting the pivotal role of some genes in the regulation of fat and skeletal muscle growth, which, in turn, affects the efficiency of feed conversion (Switoski et al., 2010). Although several single nucleotide polymorphisms (SNPs) were found significantly associated with these traits, published researches were mainly focused on cosmopolitan breeds slaughtered at a final live weight from 90 to 110 kg (Kouba and Sellier, 2011; Fan et al., 2010; Stachowiak et al., 2007; Muráni et al., 2006). Less information is available for the breeds selected for the production of Italian heavy pigs, which are slaughtered at a final live weight higher than 160 kg, as required for the Italian DOP production of high-quality and certified products like San Daniele Ham. Among the candidate genes for average daily gain, we focused on the genetic variability in the promoter region of leptin (LEP), myogenic factor 6 (MYF6), myogenic differentiation factor (MYOD1), osteopontin (OPN) and stearoyl-CoA desaturase (SCD). The myogenic factors, MYF6 and MYOD1, were selected for their physiological function in muscle development (Ropka-Molik et al., 2010) and for their involvement in the regulation of OPN expression. The OPN gene stimulates the proliferation of myoblasts and, deposited in the extracellular matrix, induces their differentiation in regulation of bone tissue deposition (Han et al., 2012). LEP and SCD were selected for their role in fat deposition (Switoski et al., 2010).

In this paper, SNPs in these target genes were investigated in relation to average daily gain (ADG) in pig populations of three different Italian pure breeds (Large White, Duroc and Landrace).

Materials and methods

Animals and sampling

The ADG (g/d) of pigs from 30 through 160 kg live weight was recorded on Italian Large White (ILW), Italian Duroc (IDU) and Italian Landrace (ILA) performance tested pigs by the National Association of Pig Breeders (Associazione Nazionale Allevatori Suini, ANAS; http://www.anas.it). Pigs were fed under a quasi ad libitum nutritive level, meaning that about 60% of pigs were able to ingest the entire supplied ration. Phenotypic data were used by ANAS to calculate the EBVs by a BLUP-multiple trait-animal model (Henderson and Quaaas, 1976). Traits were analysed by different models altogether, including the fixed effects of batch on trial, sex, inbreeding of the animal, batch at slaughtering, age at slaughtering and the random effects of animal, litter, permanent environment plus model error. For the present research, ANAS provided for the experimental animals the EBVs for Average Daily Gain (ADG-EBVs) and Random Residuals (Fontanesi et al., 2010). Random Residuals (RRs) are the errors from fixed models (one per trait) made by the fixed part of the mixed model used in the computa-
tion of EBVs for the same trait. Ideally, RR of each animal includes the statistical error plus all the random effects (genetic, litter and permanent environment). For association studies, 100 pigs with the most negative (minus-variant group: MV) and 100 pigs with the most positive values (plus-variant group: PV) of ADG-EBVs for each breed were selected (600 overall). The number of different parents (boars and sows) was 30 and 51 in ILW PV, 67 and 73 in ILW MV, 24 and 50 in IDU PV, 56 and 67 in IDU MV, 19 and 48 in ILA PV, 46 and 54 in ILA MV. The means ± standard deviations of RRs for ADG in the extreme groups were: 104.8 ± 9.1 g/d and -50.6 ± 11.2 g/d for ILW pigs (13200 sib-tested pigs); 88.3 ± 7.5 g/d and -27.6 ± 13.1 g/d for IDU pigs (6000 sib-tested pigs); 110.8 ± 10.1 g/d and -36.2 ± 12.2 g/d for ILA (5200 sib-tested pigs).

DNA extraction and analysis

Blood samples were collected from 600 pigs overall. DNA was extracted from EDTA whole blood samples by salting out procedure (Sambrook and Russell, 2001). Six SNPs in the promoter region of leptin (LEP_1 and LEP_2), myogenic factor 6 (MYF6), myogenic differentiation factor (MYOD1), osteopontin (OPN) and stearoyl-CoA desaturase (SCD) genes were investigated. Gene functions and SNP details were reported in Table 1. Genotyping of selected SNPs was performed in outsourcing, using the patented KASP SNP genotyping system (KASPar, KBioscience). This assay is a homogeneous fluorescent FRET based system. FRET, or Fluorescent Resonance Energy Transfer allows the detection of SNP’s without the need for a separation step, coupled with the power of competitive allele specific PCR. Details of the method used can be found at http://www.kbioscience.co.uk/reagents/KASP.html.

Data were analysed using the SPSS software (SPSS, 1997). Fisher’s two-tailed exact test with a multiple testing correction procedure was used to evaluate the allele frequencies distribution of the six SNPs in plus-variant (PV) and minus-variant (MV) groups of pigs for ADG in each breed. The coefficients of correlation between ADG-EBVs and relative RRs were 0.728 for ILW, 0.798 for IDU and 0.743 for ILA (P < 0.01).

Associations between the genotypes and the RRs were assessed using the general linear model (GLM) procedure of SPSS with fixed effect of the genotypes. The comparisons of the means were conducted with the least square procedure and Bonferroni correction. Putative transcription factor binding sites (TFBS) were identified using the bioinformatics tool MATINSPECTOR (Cartharius et al., 2005) of Genomatix suite.

Results and discussion

The present study was focused to the analysis of SNPs located in the promoter region of genes involved in metabolic pathways connected with pig carcass traits. Allele frequencies of the 6 polymorphisms that were genotyped for all animals are listed in Table 2. All SNPs were segregating in Italian populations except OPN, in ILW and ILA, and SCD, in ILA.

The promoter region of LEP was recently resequenced in Italian heavy pig breeds by Crisà et al. (2011), who detected 14 specific SNPs, including the two polymorphisms analysed in present study. In vitro gene reporter assays suggested a modulation of transcriptional activity of LEP, associated with polymorphisms of LEP gene in pig were associated with a variation of the feed intake, ADG and BFT other than of reproductive traits. Conflicting results have been reported in association studies of LEP polymorphisms with the same phenotypic traits, and these probably depend from the experimental population used in the study (Jiang AND Gibson, 1998; Chen et al., 2004; Syydowski et al., 2004; Stefanon et al., 2004; De Oliveira et al., 2006). Conflicting results for the SNPs of LEP gene were also observed in the present study. The two LEP SNPs segregated in the three populations, and significant differences (P < 0.05) of alleles frequencies were calculated for LEP_1 between MV and PV for ADG-EBVs. In IDU, 2% of individuals showed the A allele in PV, whilst this allele had a frequency of 23% in MV. Moreover, homozygote animals for TT genotype in PV accounted for 96% of IDU, but only 62% in MV and the RRs of ADG associated with TT genotype in MV were 2% of individuals showed the A allele in PV, whilst this allele had a frequency of 23% in MV. Moreover, homozygote animals for TT genotype in PV accounted for 96% of IDU, but only 62% in MV and the RRs of ADG associated with TT genotype in MV were observed.

Also the LEP_2 SNP segregated in the three breeds and the genotype with allele C showed significantly higher RRs for ADG in IDU (P < 0.01), lower values in ILA (P < 0.05) and no significant differences in ILW.

The variable effects of LEP_1 and LEP_2 genotypes in IDU, ILA and ILW could be related with the differences in the genetic background of the breeds and to the complex regulation that LEP exerts in several physiological pathways (Barb et al., 2001). The MYF6 and MYOD1 genes (alias MYF5) belong to the MyoD family and they code for the bHLH transcription factors, which play a key role in later stages of myogenesis, as differentiation and maturation of myotubes (Lassar et al., 1989).
Significant differences (P<0.01) of allele frequencies of MYF6 between plus and minus pigs were found for ADG-RRs in ILA breed (Table 2) and the mean values associated with genotypes for CC and TT were 75.6 g/d and 14.2 g/d (P<0.05), with heterozygote showing intermediate values (Table 3). According to Wyszysińska-Koko and Kuryl (2004), the SNP of MYF6 segregated in Polish Landrace and Polish Large White, but was associated with average daily gain only in the latter breed. From the results reported in Tables 2 and 3, it appears that the degree of association changes between the 3 Italian breeds, as result from the lack of significance between MYF6 and RRs of ADG for ILW and IDU. However, Wyszysińska-Koko et al. (2006) reported that this point mutation in the promoter region of MYF6 did not affect gene expression, but the authors concluded that these results were not exhaustive, suggesting further investigations.

Significant differences (P<0.01) of alleles frequencies of the MYOD1 gene between MV and PV were found (Table 2). The mean ADG-RRs values significantly associated with genotypes (P<0.05) indicated that the difference between AA genotype and GG genotype was equal to +73.1±21.5 g/d in IDU (Table 3), but no significant differences were found for ILW and ILA. Urbański and Kuryl (2004) reported the g.302G>A transition for MYOD1 gene did not segregate in Duroc but segregated in Large White pigs. In a previous study, Cieślicki et al. (2000) found an association of this SNP with meatiness, suggesting that the point mutation could be causative. Only limited information is available for the association of the SNPs of OPN and SCD with the RRs analysed in the present paper.

The OPN gene encodes for osteopontin, an acidic single-chain phosphorylated glycoprotein belonging to the small integrin-binding ligand N-linked glycoproteins/cytokines which undergo intensive post translation modification, including phosphorylation, glycosylation and cleavage, yielding variants of molecular weight ranging from 25 to 75 kDa (Johnson et al., 2003). Polymorphisms of OPN are known to be associated with leg and body conformation traits in pigs (Ontera et al., 2008). For OPN significant differences (P<0.01) in alleles frequencies between MV and PV were found only in IDU (Table 2) and the mean ADG-RRs values indicated that the GG genotype had a +69.8±14.1 g/d (P<0.05) compared to AA genotype (Table 3). In agreement to Muràni et al. (2009) we observed that the OPN SNP segregated only in Duroc breed (Table 2). Results of Muràni et al. (2009) revealed a lower abundance of mRNA associated with the mutant G allele of OPN, sug-

### Table 2. Distribution of LEP, MYF6, MYOD1 and OPN single nucleotide polymorphisms in three Italian pig breeds.

| SNP    | Breed | Allele | Allele frequencies | P(Fisher) |
|--------|-------|--------|--------------------|-----------|
| LEP_1  | [AT]  | ILW A  | 0.41               | 86        | 0.26 | 88 | ** |
|        |       | IDU A  | 0.02               | 99        | 0.23 | 98 | ** |
| LEP_2  | [CT]  | ILW C  | 0.57               | 89        | 0.66 | 92 | ** |
|        |       | IDU C  | 0.74               | 98        | 0.57 | 99 | ** |
| MYF6   | [CT]  | ILW C  | 0.26               | 90        | 0.33 | 92 | ** |
|        |       | IDU C  | 0.31               | 97        | 0.35 | 97 | ** |
| MYOD1  | [AT]  | ILW A  | 0.42               | 88        | 0.55 | 93 | * |
|        |       | IDU A  | 0.95               | 97        | 0.78 | 98 | ** |
| OPN    | [AV]  | ILW A  | 0.15               | 99        | 0.41 | 97 | ** |
|        |       | IDU A  | 1.00               | 95        | 1.00 | 97 | ** |
| SCD    | [CT]  | ILW C  | 0.24               | 85        | 0.16 | 94 | ** |
|        |       | IDU C  | 0.23               | 99        | 0.63 | 99 | ** |
|        |       | ILA C  | 1.00               | 95        | 1.00 | 97 | ** |

Allele frequencies distribution of the six SNPs and probability (***P<0.01; **P<0.05) from Fisher’s two-tailed exact test of equal frequency in plus-variant (PV) and minus-variant (MV) groups of pigs for ADG in Italian Large White (ILW), Italian Duroc (IDU) and Italian Landrace (ILA). Fisher’s two-tailed exact test with a multiple testing correction procedure was used.

### Table 3. Association analysis.

| SNP    | Breed | Homozygote1 | Homozygote2 | P-value |
|--------|-------|-------------|-------------|---------|
| LEP_1  | [AT]  | ILW 47.28  | 18.05       | 45.12   | 8.56   | 80 |
|        |       | IDU -21.26 | 1.27        | -12.48  | 7.04   | 33 |
|        |       | ILA 12.63  | 16.32       | 23.66   | 8.99   | 67 |
| LEP_2  | [CT]  | ILW 6.15   | 9.40        | 37.20   | 8.19   | 91 |
|        |       | IDU 46.21  | 6.12        | 18.33   | 6.46   | 61 |
|        |       | ILA 55.04  | 7.68        | 33.65   | 8.86   | 71 |
| MYF6   | [CT]  | ILW -8.00  | 20.70       | 26.84   | 8.82   | 81 |
|        |       | IDU 24.77  | 16.61       | 27.02   | 5.89   | 102 |
|        |       | ILA 75.58  | 19.59       | 52.59   | 7.53   | 95 |
| MYOD1  | [AA]  | ILW 2.51   | 10.90       | 28.70   | 8.69   | 83 |
|        |       | IDU 41.20A | 4.17       | 2.45    | 8.25   | 38 |
|        |       | ILA -1.00  | 17.65       | 28.16   | 8.69   | 67 |
| OPN    | [AA]  | ILW 25.81  | 5.85        | -0.00   | -0.00  | -0.00 |
|        |       | IDU -17.82 | 7.47        | 13.36   | 6.62   | 74 |
|        |       | ILA 36.59  | 5.39        | 13.17   | -0.00  | -0.00 |
| SCD    | [CT]  | ILW 65.50  | 25.26       | 29.90   | 10.76  | 52 |
|        |       | IDU -13.11 | 5.86        | 26.04   | 6.47   | 79 |
|        |       | ILA 36.59  | 5.39        | 13.17   | -0.00  | -0.00 |

Least square means (LSM) of random residuals (g/d) for average daily gain with their standard errors (SE) for each genotype in Italian Large White (ILW), Italian Duroc (IDU) and Italian Landrace (ILA). The number of pigs genotyped for each group was reported in brackets. The comparisons of the means were conducted with the least square procedure and Bonferroni correction. LSM different at P<0.01; **LSM different at P<0.05.
gesting that this point mutation could be considered as a DNA-marker for association studies of muscle and growth traits. However, Muráni et al. (2009) reported that the same SNP in OPN promoter induces an aberrant splicing, although does not change the primary structure of the osteopontin protein. This aberrant splicing may counteract the negative effect of the G allele on mRNA expression of OPN by enhancing translational efficiency or RNA stability. The associated higher values of ADG-RRs (P<0.01; Table 3) with G allele in IDU agree with a major positive phenotypic effect of this point mutation. Stearoyl-CoA desaturase is a microsomal membrane bound, iron-containing enzyme required for the biosynthesis of unsaturated fatty acids (Uemoto et al., 2012). As for the OPN gene, for the SCD gene significant differences (P<0.01) in alleles frequencies between MV and PV were found only in IDU (Table 2). For this breed, the mean ADG-RRs values for the CC genotype was -13.1±5.9 g/d compared to this breed, the mean ADG-RRs values for the CC genotype at 6.1 g/d for TT (P<0.01), with heterozygote showing intermediate values (Table 3). For SCD, Ren et al. (2004) reported high polymorphism levels of the g.2228C>T SNP only in Duroc pigs, while the C allele frequency was very low for Large White and Landrace breeds. However, Ren et al. (2004) failed to find an association between this SNP and carcass traits and the discrepancy is probably due to the different selection schemes applied for Italian Duroc breed in comparison to those reported by Ren et al. (2004).

Promoters were searched for putative TFBS in the flanking regions of targeted SNPs using MATINSPECTOR software tool. MATINSPECTOR database locates putative binding domains based on sequence similarity. The analysis identified a large number of putative recognition sites, several of them absent on sequence analysis identified a large number of putative domains based on sequence similarity. The TFBS of which SNPs are targets represented by the matrix family. The tissue specific splicing of the first intron is located in the core sequence of TFBS, even though the mutation does not seem to affect the affinity. The analysed g.3836A>G transition of OPN is located in an evolutionarily conserved region of the promoter and affects the gene function by activating aberrant splicing of the first intron (Muráni et al., 2009), thus representing an interesting DNA-marker to study phenotypic effects. For the remaining matrices of the investigated promoter regions the evidences of their role with specific tissue functions are not available yet. However, these hints require being further investigated performing functional studies on the different promoter alleles by using in vitro gene reporter assays.

**Conclusions**

Regulatory regions represent a very interesting target for research due to their effect on gene expressions and, potentially, on phenotypes. Gene variants of LEP, MYF6, MYOD1, OPN and SCD were investigated to evaluate their association with traits under selection in Italian breeds. The Italian selection of pig breeds is aimed to improve the traits related to organogenesis and morphogenesis as well as in cellular proliferation and differentiation during animal development (Buonamici et al., 2003). Binding sites for CEBP were found in promoter regions of LEP, OPN and MYOD1 genes. The CEBPs are members of the basic-leucine zipper class of transcription factors and play roles in adipocyte differentiation and adipocyte gene expression (Manupur et al., 1997). They act as homo- or heterodimers and their tissue distribution is not limited to adipose tissue (Lekstrom-Himes & Xanthopoulos, 1998). However, regulation of the expression of several CEBP family members is reported during adipogenesis, and recent gain and loss of function studies indicate that these proteins have a considerable impact on fat cell development (Dodson et al., 2010). The SNP investigated for the regulatory element of CEBP present in the MYOD1 is located in the conserved consensus sequence of TFBS, even though the mutation does not seem to affect the affinity. The analysed g.3836A>G transition of OPN is located in an evolutionarily conserved region of the promoter and affects the gene function by activating aberrant splicing of the first intron (Muráni et al., 2009), thus representing an interesting DNA-marker to study phenotypic effects. For the remaining matrices of the investigated promoter regions the evidences of their role with specific tissue functions are not available yet. However, these hints require being further investigated performing functional studies on the different promoter alleles by using in vitro gene reporter assays. Economically important traits. These base mutations can lead to a modification of the consensus sequence for a large number of transcription factors, altering gene expressions and their functions. However, evidences of a different transcriptional modulation required to be ascertainment and investigations on transcriptional activity of mutated genes are needed to understand if these SNPs are causative mutations or if they are in linkage disequilibrium with others.

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## APPENDIX

### Appendix Table 1. Putative General Core Promoter Elements. TFBS of which SNPs led to a putative effect on the recognition of the target sequence were reported. The allele that create an additional core sequence was specified for each site, while the same TFBS was absent with alternative allele.

| Gene/factor | Allele | Strand | Sequence |
|-------------|--------|--------|----------|
| LEP_1       |        |        |          |
| Bicoid-like homeodomain transcription factors | T | + | ttgCTAAaatccaaaat |
| snRNA-activating protein complex | T | + | cggatCCATggtgcaaat |
| Octamer binding protein | A | - | gttTGCAatgctatt |
| LEP_2       |        |        |          |
| Ccaat/Enhancer Binding Protein | C | - | cggagagAGAcgacc |
| EVII-myleoid transforming protein | C | - | cggagagGATGagggaaa |
| GATA binding factors | T | - | agegGATAGagga |
| MYF6        |        |        |          |
| Bicoid-like homeodomain transcription factors | T | + | aggtTAATcccggtt |
| Hepatic Nuclear Factor I | T | + | tGTAAttccgggt |
| C-abl DNA binding sites | A | - | aaATCTaccag |
| Bicoid-like homeodomain transcription factors | C | + | aggtTAATcccggtt |
| PAX-4/PAX-6 paired domain binding sites | C | + | aggCCTatggtgcaag |
| Grainhead-like transcription factors | C | + | atgccGGTGcgtt |
| MYOD        |        |        |          |
| Krueppel like transcription factors | G | + | gcccagcGGGTTgggca |
| Core promoter motif ten elements | A | - | caattccAGAgcagaggctg |
| CCAAT binding factors | A | - | ttgGCAATTccacgg |
| OPN         |        |        |          |
| Signal transducer and activator of transcription | G | - | gattttGTC44aatct |
| cAMP-responsive element binding proteins | A | - | aaaaatGTTAAtatat |
| Ccaat/Enhancer Binding Protein | A | - | aattGTC44agaa |
| PAR/AZIP family | A | - | aaataatGTC44agaa |
| Signal transducer and activator of transcription | A | - | gattTTGGC1eaaaaat |
| SCD         |        |        |          |
| v-ERB and RAR-related orphan receptor alpha | C | + | ggcagttaagGACAcgagc |
| Basonuclein rDNA transcription factor Poll | C | - | taggagagcGTTAcgta |
| PAX-5 B-cell-specific activator protein | C | + | acagcGCAacgagcagcagtcct |

In red, SNP affecting binding; in italics underlined, position with high matrix conservation, according to MATINSPECTOR results; capital letters, core sequence.