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Prospecting polymorphisms in the *PPP3CA* and *FABP4* genes and their association with early pregnancy probability in Nellore heifers

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ABSTRACT

Early pregnancy probability (P16) in heifers is a trait of high economic value for beef cattle production. Early pregnancy is defined based on the conception and calving of a heifer given that the animal had entered the breeding season at about 16 months of age and is considered a strong predictor of puberty in Nellore cattle. The aim of this study was to identify polymorphisms in the protein phosphatase 3 – catalytic subunit, alpha isozyme (*PPP3CA)* and fatty acid-binding proteins 4 (*FABP4)* genes, which have been associated with P16. The exon regions of the candidate genes were sequenced in 380 heifers. Two polymorphisms were detected in *PPP3CA* and 13 in *FABP4*. A deletion (rs134413439) was identified in the *FABP4* gene in all samples, which could be a variation that occurs across breeds since the reference genome is from a Hereford cow. Two SNPs in the *FABP4* gene were associated with P16 after Bonferroni correction. However, none of the haplotypes exerted a significant effect ($P > 0.05$). This study showed that the *PPP3CA* and *FABP4* genes are polymorphic in Nellore cattle. Furthermore, the *FABP4* gene was found to be associated with early pregnancy in heifers, reinforcing the contribution of lipid metabolism to reproduction.

1. Introduction

Reducing the age at first conception and first calving promotes improvement of reproductive efficiency and has a major impact on the efficiency of beef cattle production systems (Beretta et al., 2001; Fortes et al., 2010; Teixeira et al., 2002). Furthermore, this reduction positively influences genetic progress by decreasing the generation interval and permitting to increase the intensity of selection (Van Melis et al., 2010). Early pregnancy probability (P16) is considered a good indicator of age at puberty and might be used as a selection criterion. However, since this trait is expressed late and only in females, it has been little used in breeding programs not only in Brazil but also in the rest of the world (MacNeil et al., 2006).

In an attempt to identify the association between known polymorphisms in genes related to adipose tissue and P16 in Nellore heifers, Dias et al. (2015) used data from 1689 precocious (calvings up to 18 months) and non-precocious heifers genotyped with the High-Density Bovine SNP BeadChip (Illumina, Inc). Fifty-seven candidate genes and 443 SNPs were analyzed. The authors found a significant effect of haplotypes located in the protein phosphatase 3 – catalytic subunit, alpha isozyme (*PPP3CA*) and fatty acid-binding proteins 4 (*FABP4*) genes on P16 and concluded that these genes exert an influence on this trait.

Among many factors, the influence of adipose tissue on P16 is due to the fact that this tissue is one of the main sites of sex steroid metabolism (Kershaw and Flier, 2004). Taniguchi et al. (2008) found the *PPP3CA* gene to be differentially expressed and it is down-regulated during the adipogenesis. Furthermore, Martin et al. (2007) observed that blockade of this gene caused infertility in male rats.

The *FABP4* gene is a member of the FABP family which are fatty acid-binding proteins. The gene is located on bovine chromosome 14. Studies have reported an association of this gene with variations in cholesterol percentage (Hoashi et al., 2008). Michal et al. (2006) demonstrated a key role of FABP4 in the absorption, transport and metabolism of fatty acids. Free fatty acids that are transported by FABP4 can be activated and oxidized to produce acetyl-CoA and NADH. Part of this acetyl-CoA is used for the biosynthesis of cholesterol which, in turn, is the precursor of steroid hormones (Berg et al., 2002). Steroids are

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involved in the differentiation, growth and physiology of reproductive organs (Havlíková et al., 2006; Pearce and Jordan, 2004).

The objective of the present study was to identify polymorphisms in the exon regions of the *PPP3CA* and *FABP4* genes and to evaluate their possible association with P16 in heifers.

2. Material and methods

2.1. Animals

The data and samples used in the present study were collected from 2036 Nellore heifers born in the years 2007 and 2008 and exposed to bulls in the anticipated breeding seasons of 2009 and 2010 on farms belonging to the company Agropecuária Jacarezinho Ltda., State of Bahia, Brazil. Data regarding pregnancy (success or failure) were obtained, as well as information about the pedigree, register and management of these animals. In addition, hair follicles were collected from the tail for DNA extraction.

On these farms, the animals were exposed to bulls during two breeding seasons. An anticipated breeding season lasting 60 days occurs between February and April to identify animals that reach puberty early. All heifers aging 16–18 months were exposed, regardless of weight and body condition. The mating system for heifers is a controlled breeding, with the ratio of 1:50 bull per heifers. Approximately, 60 days after the end of the anticipated breeding season, heifers were submitted to rectal palpation for the evaluation of pregnancy. Heifers that did not conceive in the anticipated breeding season are used in the cow breeding season of the subsequent year when they are about two years of age.

During the step of data preparation, inconsistent data resulting from registration errors were eliminated. The contemporary groups (CG) were formed considering all effects shared by the animals and always maintaining connectedness among groups. For P16, the CG were defined by farm, year and season of birth of the heifers. The CG containing less than ten animals and those without variation in the trait (all females became pregnant or all females were empty) were excluded. After analysis of consistency, 1689 heifers remained and the rate of early pregnancy at 16 months was 30.52%. Of these, 380 heifers were selected for sequencing of the candidate gene regions based on the pregnancy rate of the CG, considering a minimum rate of 30%. Thus, the 380 females selected belonged to 19 CG and the rate of early pregnancy of the 380 heifers was 40.26%.

2.2. Candidate genes

Based on the position of the haplotypes described by Dias et al. (2015), exon regions that flank or a located within haplotypes exhibiting significant effects at the 5% level were delimited. Thus, the four exons of the *FABP4* gene and exons 2 and 3 of the *PPP3CA* gene were used. Fig. 1, adapted from Ensembl (Cunningham et al., 2015), illustrates the haplotypes described by Dias et al. (2015) and the exons selected for sequencing.

Table 1
Region per amplicon containing the exon/intron of the candidate genes.

Gene	Chr	Location		Length (bp)
		Start	End	
PPP3CA	6	24,812,677	24,812,991	314
PPP3CA	6	24,985,650	24,985,860	211
PPP3CA	6	25,060,198	25,060,332	135
FABP4	14	46,833,660	46,833,886	227
FABP4	14	46,834,340	46,834,451	112
FABP4	14	46,835,034	46,835,216	183
FABP4	14	46,837,916	46,838,058	143

Chr: chromosome.

2.3. DNA extraction, customized panel of MiSeq sequencing, and detection of variants

DNA was extracted from the caudal hair follicle by the phenol-chloroform-isoamyl alcohol method (Sambrook et al., 1989). A custom panel was built with the TruSeq Custom Amplicon® Kit (Illumina, Inc) using the DesignStudio software (available at: http://designstudio.illumina.com/) based on the position of the exons. For full coverage of the exon region, part of the introns and intergenic region was used. The size of the amplicon used for customization was 425 bp, ensuring 100% coverage of the delimited region of each exon (Table 1).

An amount of 250 ng DNA was used to generate amplification products covering the regions. Indexed adapters were added to the ends of the DNA amplicons by limited cycle PCR. The DNA libraries obtained were validated in an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). Next, the DNA libraries were sequenced (2 × 250 paired-end) with the Illumina MiSeq® System using the MiSeq Reagent Kit v2 (500 cycles) according to manufacturer instructions. Image analysis and base calling were performed using the MiSeq software.

The sequences in FASTQ format were aligned with the UMD3.1. bovine reference genome through BaseSpace® (available at: https://basespace.illumina.com/) (Illumina, Inc.) using the TruSeq Amplicon tool. The same tool was employed for the detection of variants using the somatic option. Only variants with a minor allele frequency (MAF) > 5% were considered. Gene annotation was performed with Ensembl and only variants with a Phred quality score ≥ 40 were considered.

2.4. Allele and Genotype Frequencies and Hardy-Weinberg Equilibrium

Descriptive statistics were generated with the Plink software (Purcell et al., 2007). Allele and genotype frequencies of the SNPs were calculated as the ratio between the number of a certain allele and the total number of alleles (Falconer and Mackay, 1996). Possible deviations of the observed genotype frequency from the expected frequency were tested by the chi-square test in order to determine whether the locus of interest was in Hardy-Weinberg equilibrium (HWE) in the population sample studied (Falconer and Mackay, 1996).

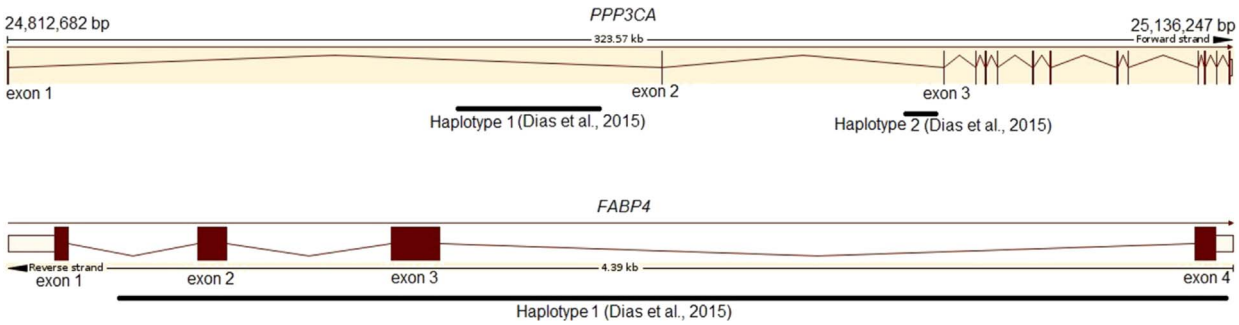

Fig. 1. Schematic diagram, adapted from Ensembl, illustrating the position of haplotypes with significant effects (Dias et al., 2015) and the exons selected for sequencing.

Table 2
Polymorphisms in the regions of the candidate genes amplified by the MiSeq® technology (Illumina, Inc.).

Gene	Chr	SNP_ID	Position	Mutation	Region	Consequence	MAF
PPP3CA	6	rs110138405	24985900	C/G	intron 2		0.09079
PPP3CA	6	rs452520974	25060456	T/C	intron 3		0.3066
FABP4	14	rs109593774	46833649	G/C	downstream		0.1197
FABP4	14	rs110266999	46833693	T/C	3′UTR		0.1263
FABP4	14	rs109077068[a]	46833778	G/A	3′UTR		0.1237
FABP4	14	rs449843560	46833805	C/T	3′UTR		**0.01316**
FABP4	14	rs110370032	46833834	T/C	exon 4	Synonymous	0.1316
FABP4	14	rs110383592	46834345	C/G	exon 3	Synonymous	**0.03553**
FABP4	14	rs110652478	46834365	C/T	exon 3	Synonymous	**0.001316**
FABP4	14	rs109014985	46834523	T/A	intron 2		0.1368
FABP4	14	rs134173517	46834560	T/G	intron 2		0.1368
FABP4	14	rs134413439	46835006	T/-	intron 2		–
FABP4	14	rs110757796	46835065	T/C	exon 2	Non-synonymous	0.07237
FABP4	14	rs109316505	46835220	G/C	intron 1		**0.006579**
FABP4	14	rs109682576	46838280	T/C	upstream		0.05789

Chr: chromosome; MAF: minor allele frequency.
[a] SNP also genotyped with the High-Density Bovine SNP BeadChip (Illumina, Inc.).

2.5. Analysis of linkage disequilibrium

Linkage disequilibrium (LD) between SNPs was estimated based on r^2 statistic (Hill and Robertson, 1968) using the Plink software (Purcell et al., 2007). Considering two loci with two alleles per locus (A1/A2 and B1/B2), the following formula was used (Hill and Robertson, 1968):

$$r^2 = \frac{D^2}{[f(A1)*f(A2)*f(B1)*f(B2)}$$

where fA1, fA2, fB1 and fB2 are the frequencies of alleles A1, A2, B1 and B2, respectively, and D^2 = f(A1_B1)*f(A2_B2) - f(A1_B2)*f(A2_B1), with fA1_B1, fA2_B2, fA1_B2 and fA2_B1 being the frequencies of haplotypes A1_B1, A2_B2, A1_B2 and A2_B1 of the population, respectively (Hill, 1981).

2.6. Quality control of genotypes

Quality control analysis of the genotypes for the 2035 samples genotyped with the panel of 777,000 SNPs of the High-Density Bovine SNP BeadChip (Illumina, Inc.) and the 380 samples sequenced with the MiSeq® technology (Illumina, Inc.) was performed using the Plink v1.07 software (Purcell et al., 2007). SNPs with a call rate < 95%, MAF < 0.05 and p-value for HWE less than 10^{-6} were eliminated. SNPs found in the region from 25,000,000 to 26,000,000 bp of chromosome 6 and in the region from 46,000,000 to 47,000,000 bp of chromosome 14, regions where the candidate genes are located, were considered. For quality control of the samples, animals with a call rate < 90% were excluded.

Among the 2035 females used for imputation, 29 were excluded by quality control of the samples, i.e., they had a call rate < 90%. A total of 609 SNPs from the High-Density Bovine SNP BeadChip (Illumina, Inc.) were selected from the chromosomes of the two genes studied. Of these, 265 were excluded because of a MAF < 0.05; 14 had a p-value for HWE < 10^{-6} and 19 had a call rate < 0.95. Thus, the information of 311 SNPs was used for genotype imputation, with 194 SNPs being located on chromosome 6 and 117 SNPs on chromosome 14. Thirty of these SNPs were found in the region of the *PPP3CA* gene and two in the region of the *FABP4* gene. The SNPs of the panel located within the region of the genes, as previously described, were also used for statistical analysis.

2.7. Genotype imputation and haplotype reconstruction

To increase the number of individuals analyzed, polymorphic genotypes identified by MiSeq® sequencing (Illumina, Inc.) for samples that were not sequenced but were genotyped with the High-Density Bovine SNP BeadChip (Illumina, Inc.) were imputed using the Fimpute software (Sargolzaei et al., 2014). The same software was used to estimate the phase of ligation and construction of haplotypes formed by four SNPs, including rs110370032 and rs110652478 located in the exon and rs110266999 and rs109077068 located in the 3′ untranslated region (3′UTR) of the *FABP4* gene.

2.8. Statistical analysis

Early pregnancy was defined based on the conception and calving of a heifer given that the animal had entered the breeding season at about 16 months of age. Value 1 was attributed to heifers that calved at less than 31 months (success) and value 0 to those that did not (failure).

For analysis, a univariate mixed linear model was applied using the GEMMA software (Zhou and Stephens, 2012). The effect of the genotypes identified and imputed for 1660 heifers on P16 was estimated using the following statistical model:

$$y = W\alpha + x\beta + u + e$$

where y = phenotypes for trait P16 for the 1660 heifers; W = incidence matrix of fixed effects (CG); α = vector corresponding to the fixed effects, including the intercept; x = vector containing the genotypes of the SNPs or haplotypes identified for each animal; β = effect of each SNP or haplotype; u = vector of random additive genetic effects (polygenic), and e = random error.

It is assumed that $u \sim (0, \sigma^2 aK)$, where K is the genomic kinship relatedness matrix, and σ_a^2 is the additive genetic variance (polygenic). Regarding the residual effect, it was assumed that $e \sim (0, \sigma_e^2 In)$, where e is an error, In is an identity matrix, and σ_e^2 is the residual variance.

The alternative hypothesis, H1:$\beta \neq 0$, against the null hypothesis, H0: $\beta = 0$, for each SNP was evaluated by the Wald test (p-wald). Bonferroni correction for multiple comparisons was applied to ensure the maintenance of the error rate of the statistical tests in the experiment.

3. Results

Fifteen polymorphisms, two in the *PPP3CA* gene and 13 in the *FABP4* gene, were identified in the regions amplified in the 380 Nellore heifers (Table 2). All polymorphisms had a Phred quality score higher than 40, corresponding to a probability of incorrect base call of 0.001–0.01 (Illumina, 2011).

Only four SNPs were located within an exon region, all of them in the *FABP4* gene, with only one changing the amino acid (rs110757796).

Table 3
Allele and genotype frequencies and Hardy-Weinberg equilibrium for SNPs identified in the regions of the candidate genes.

Chr	Polymorphism	Allele frequency		Genotype frequency			p-value (HWE)
		G	**C**	**GG**	**GC**	**CC**	
6	rs110138405	0.09079	0.90921	0.00526	0.17105	0.82368	0.75490
14	rs109593774	0.11973	0.88026	0.00526	0.22895	0.76579	0.13930
14	rs110383592	0.03553	0.96447	0.00789	0.05526	0.93684	0.00828
14	rs109316505	0.00658	0.99342	0.98684	0.01316	0	1
		T	**C**	**TT**	**TC**	**CC**	
6	rs452520974	0.30657	0.69342	0.07632	0.46053	0.46316	0.11780
14	rs110266999	0.12631	0.87368	0.00263	0.24737	0.75	0.01752
14	rs449843560	0.01316	0.98684	0.01053	0.00526	0.98421	**0.000000014***
14	rs110370032	0.13157	0.86842	0.00526	0.25263	0.74211	0.04218
14	rs110652478	0.00132	0.99868	0	0.00263	0.99737	1
14	rs110757796	0.07237	0.92763	0.00263	0.13947	0.85789	0.7082
14	rs109682576	0.05789	0.95211	0.01579	0.08421	0.90000	0.0006638
		T	**A**	**TT**	**TA**	**AA**	
14	rs109014985	0.13684	0.86315	0.00526	0.26316	0.73158	0.027750
		T	**G**	**TT**	**TG**	**GG**	
14	rs134173517	0.13684	0.86315	0.00263	0.26842	0.72895	0.004301
		G	**A**	**GG**	**GA**	**AA**	
14	rs109077068	0.12368	0.87631	0.00263	0.24211	0.75526	0.017640

Chr: chromosome; HWE: Hardy-Weinberg equilibrium.

* Significant at $p < 10^{-6}$.

The other SNPs were located within the 3′UTR of the *FABP4* gene, in introns of the *PPP3CA* and *FABP4* genes, and in the intergenic region (Table 2). The rs109077068 SNP is part of the High-Density Bovine SNP BeadChip (Illumina, Inc.) and is found in the haplotype block described by Dias et al. (2015). A deletion was found in the intron region of the *FABP4* gene (rs134413439) when compared to the homologous region of the bovine genome.

Four SNPs (rs449843560, rs110383592, rs110652478 and rs109316505) were excluded after quality control because of a MAF < 0.05 (Table 2). As can also be seen in Table 2, the MAF was higher than 0.10 only for SNP rs452520974, which was the only SNP that had two alleles with intermediate frequencies. After imputation, the only SNP excluded by quality control was rs109682576 because of a MAF of 0.04127764.

The allele and genotype frequencies and p-values for HWE obtained for the 380 animals sequenced (Table 3) showed that only SNP rs449843560 was not in HWE (P < 0.000001) and was excluded from the subsequent analyses. The same SNP had already been removed because of a MAF < 0.05. Of the remaining nine SNPs, only two present in the *FABP4* gene were located within the exon region.

For the calculation of LD, only SNPs identified by sequencing and that passed quality control were considered. For the two SNPs found in the *PPP3CA* gene, rs110138405 and rs452520974, the estimated r^2 was 0.0228. Table 4 shows the r^2 values between SNPs passing quality control found in the *FABP4* gene.

For association analysis, all SNPs identified that passed quality control were considered one at a time. After Bonferroni correction, only two SNPs (rs109014985 and rs134173517) in the *FABP4* gene exerted a significant effect (P < 0.05) for P16 (Table 5). After Bonferroni correction, association analysis revealed no significant effect (P > 0.05) of the haplotypes in the *FABP4* gene for P16 in Nellore heifers.

Table 5
P-value of the SNPs identified that exerted a significant effect on early pregnancy probability in Nellore heifers.

Gene	Chr	rs	Allele 1	Allele 2	p-wald
PPP3CA	6	rs110138405	G	C	0.38927240
PPP3CA	6	rs452520974	T	C	0.75901610
FABP4	14	rs109593774	G	C	0.12036960
FABP4	14	rs110266999	T	C	0.10526550
FABP4	14	rs109077068	G	A	0.08571890
FABP4	14	rs110370032	T	C	0.21835830
FABP4	14	rs109014985	T	A	**0.003752550**
FABP4	14	rs134173517	T	G	**0.004849945**
FABP4	14	rs110757796	T	C	0.15214300

Chr: chromosome.

4. Discussion

After previous genome associations studies that identified *PPP3CA* and *FABP4* as important genes for reproductive traits in indicine beef cattle, the present results pinpoint the putative causative mutations after the fine-mapping.

The only missense SNP detected in the gene *FAPB4* (rs110757796) is a conservative mutation that causes a phenylalanine (Phe) to leucine (Leu) substitution. However, an amino acid substitution can alter the configuration of the protein and affect its biological function. Although non-synonymous mutations cause greater changes in proteins,

Table 4
Estimated r^2 values for SNPs found in the *FABP4* gene.

SNP	rs109593774	rs110266999	rs109077068	rs110370032	rs109014985	rs134173517	rs110757796	rs109682576
rs109593774	–	0.856738	0.88079	0.790571	0.683691	0.679272	0.398607	0.000058
rs110266999		–	0.919944	0.848878	0.714244	0.732668	0.422842	0.000004
rs109077068			–	0.873585	0.736361	0.732388	0.413219	0.000004
rs110370032				–	0.676065	0.693504	0.389616	0.000085
rs109014985					–	0.974854	0.349988	0.000268
rs134173517						–	0.378712	0.000275
rs110757796							–	0.000054
rs109682576								–

synonymous mutations can affect the transcription rate or transcripts turnover (Flint and Woolliams, 2008) and thus interfere in the expression of phenotype.

The SNPs found in the 3′UTR region and in introns might be in an hybridize or production region of miRNA, respectively. If located in an hybridize region, these SNPs can affect gene translation and, if located in a production region, they can affect the translation of other genes (Jin and Lee, 2013). The SNPs in the intergenic region upstream of the gene may affect a transcription factor binding site (Vinsky et al., 2013). SNPs located in the 3′UTR can also influence the stability of messenger RNA (mRNA) (Matoulkova et al., 2012).

The deletion detected in the intron region of the *FABP4* gene (rs134413439 was present in all animals, it is possibly fixed in the Nellore breed. The reference bovine genome was constructed by sequencing a Hereford animal (Elsik et al., 2009), and this deletion may therefore represent a difference between the two breeds, Hereford and Nellore. There are no studies in the literature reporting an association of this deletion with any trait.

The LD between SNPs is classified as moderate to high when $r^2 > 0.2$ (Bohmanova et al., 2010), when the LD is moderate to high means that these SNPs are commonly inherited together. R^2 values < 0.2 indicate independent segregation of SNPs. The r^2 values of all SNPs with SNP rs1096682576 are less than 0.2, indicating that these SNPs will segregate independently. Qanbari et al. (2014) compared the pattern of LD between sequenced samples (n = 43) and samples genotyped with 50k and 700k chips (n = 1293) obtained from Simmental cattle. The authors observed a greater decline in r^2 values as a function of distance in sequenced samples compared to genotyped samples. According to the authors, the fact that the SNPs are close does not mean that they are linked and these SNPs may segregate independently. A decline in r^2 was observed with increasing distance of the SNPs from SNP rs1096682576.

The SNPs in the *FABP4* gene that exerted a significant effect on P16 have been associated with fat thickness and marbling (Blecha et al., 2015). Goszczynski et al. (2017), analyzing different cattle breeds, found an association of five SNPs with fat thickness and fatty acid composition. RNA sequencing studies have shown that the *FABP4* gene is a tissue specific gene expression in cattle during the puberty (Cánovas et al., 2014) and induces adipogenesis (Zhang et al., 2017). Thus, this gene probably exerts a pleiotropic effect on fertility and meat quality traits.

Owens et al. (1993) discussed that retarded body growth and fat deposition can delay puberty. Furthermore, some studies have shown the presence of transcripts of reproductive hormones in adipose tissue of different species (Barb et al., 2005; Flanagan et al., 2009; Hausman et al., 2008). Caetano et al. (2013) reported a positive genetic correlation (0.10 ± 0.11) between age at first calving and mature weight of females, thus demonstrating that selection for mature weight can influence age at first calving.

This is the first study that detected these polymorphisms in the Nellore breed and that tried to establish association between these SNPs and early pregnancy probability in Nellore heifers.

5. Conclusions

This study showed that the *PPP3CA* and *FABP4* genes are polymorphic in the Nellore breed. Studies prospecting causal mutations are important to better understand how these genes influence traits of interest. The two SNPs in the *FABP4* gene that exert a significant effect on P16 could be used to design low-density chips for the selection of animals in breeding programs.

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