GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Comparative study of leptin and leptin receptor gene expression in different swine breeds

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Genet. Mol. Res. 13 (3): 7140-7148 (2014)
Received June 25, 2013
Accepted December 5, 2013
Published February 14, 2014
DOI http://dx.doi.org/10.4238/2014.February.14.15

ABSTRACT. Leptin is an important regulator of appetite, energy metabolism, and reproduction and is mainly synthesized in the adipocytes and then secreted into the bloodstream. The leptin receptor was classified as type I cytokine receptor due to its structural homology with IL-6 receptors and the signaling pathways in which they are both involved. The aim of our study is to comparatively assess the gene expression levels of leptin (*lep*) and leptin receptor (*lepr*) in different swine breeds specialized either in meat production (Duroc, Belgian Landrace, Large White, Synthetic Lines LS-345, and LSP-2000) or fat production (Mangalitsa) in order to correlate them with morphological and productivity characteristics. Additionally, *lepr* pattern of expression was evaluated comparatively between different tissue types in the Mangalitsa breed. Our results revealed high expression of the *lep* gene in Mangalitsa compared to those of all the other breeds, while for the *lepr* gene, average/medium levels were registered in Mangalitsa and increased pattern of expression was found in the synthetic lines LS-345 and LSP-2000. Regarding the comparative analysis of *lepr* gene expression in various tissues in the Mangalitsa breed, elevated levels were found in the liver and kidney, while the lowest expression was identified in the brain and muscles. Our results suggest that the

Mangalitsa population exhibits leptin resistance, which might be correlated with atypical morpho-productive characteristics for this breed, such as below-average prolificacy and a strong tendency to accumulate fat.

Key words: Swine; Leptin; Leptin receptor; Gene expression

INTRODUCTION

Studying different human diseases by using animal models has played an important role in biomedical research and in the development of new therapies. In terms of body composition, human and swine species are similar (Mitchell et al., 2000): both display a tendency toward obesity in adulthood. Thus, pigs are suitable animal models to study obesity and obesity-related diseases. Leptin gene (*lep*) was discovered in 1994 by Zhang and collaborators (1994), generating a strong focus on its research and use as a physiological marker in obesity-associated maladies. Leptin plays an essential role in processes related to energy consumption and the amount of ingested food, thereby influencing mass and body composition (Perez-Montarelo et al., 2012). In swine populations, the leptin gene is located on chromosome 18 and consists of 3 exons and 2 introns (Bidwell et al., 1997).

The leptin receptor was discovered in 1997 by Zhang et al. during cloning studies and was classified as type I cytokine receptor. The leptin receptor family includes at least 6 isoforms arising from an alternative splicing process; thus, the leptin receptor gene (*lepr*) has multiple forms encoded by different transcripts. Leptin receptor isoforms include a long form; 4 short forms, which are distinct due to the length of the cytoplasmic region; and a soluble circulating form, which is found in the plasma (Tartaglia et al., 1995). *lepr* has been considered as a genetic marker associated with body composition, growth rate, or obesity in some swine breeds (Perez-Montarelo et al., 2012).

Multiple swine breeds specialized in meat production, as well as Mangalitsa, one of the last unimproved races dedicated to fat production, were used as models in our study.

Mangalitsa breed is part of the European local breeds that are considered to be descendants of wild boars (Georgescu et al., 2012). In addition to its specialization in the production of fat, Mangalitsa has another interesting feature, namely, low, below average prolificity (about 6 piglets per farrow), making it an ideal animal model for the study of obesity. Originally famous for fat production, the Mangalitsa breed has become important for the production of sausage products. The meat is stuffed with fat (intramuscular fat) so it is tasty and less dry than the meat from other breeds.

Large White breed was developed in Yorkshire, England, and the first imports of swines to other countries were initiated in this country, especially in the first half of the twentieth century. At present, Large White is available globally, making it one of the international breeds of swine. Over the years, specimens have been selected for meat and reproductive qualities. The Landrace breed is extremely variable: specimens show distinct characteristics depending on the aim of each selection program. Thus, breed characteristics vary from Belgian Landrace with an increased muscle mass to the Norvegian Landrace, with a massive carcass and very late maturation. The Landrace breed has a high intrinsic genetic variability, which facilitates its use in different selection programs and for the generation of swine synthetic lines. The Duroc breed specimen selection was mainly focused on the speed of growth

and accumulation of a low fat percentage at the carcass level (Schwab et al., 2007). This breed is of interest mainly because of its growth performances. Carcass quality varies when the muscle tissue percentage reaches 55%. The synthetic line 345-Periş (LS-345) was created between 1975 and 1987, in order to obtain a swine line adapted to intensive operating conditions and characterized by a high percentage of muscle tissue. Three breeds contributed to the formation of LS-345: Duroc (36.5%), Hampshire (7.5%), and Belgian Landrace (56%). This synthetic line exhibits superior qualities compared to those of the original breeds, in terms of high prolificacy, growth performance, and hull. Synthetic line LSP-2000 (LSP-2000) was created by crossing LS-345 Periş and Pietran, in order to obtain boars with increased muscle percentage. Specimens in this line are robust and exhibit high reproductive performance, and the hull is composed of more than 60% muscular tissue.

The Large White breed shows the fastest daily weight gains, reaching large volumes in relatively short periods of time. Further, this breed is also associated with a significant prolificacy (Silveira et al., 2008). However, all other swine breeds and synthetic lines analyzed in this study were improved in order to increase muscle mass percentage of the hull. They are specialized in meat production and are characterized by a rapid accumulation of muscle mass. A strong tendency for fat accumulation was observed in the Mangalitsa breed. However, due to the presence of high amounts of intramuscular fat, the flesh of this particular swine breed was more appreciated in terms of gastronomy.

Taking into account i) the major morpho-physiological changes that occur in the Mangalitsa breed compared to those occurring in the breeds dedicated for meat production, ii) the anatomo-physiological similarities between swines and humans, and iii) the fact that the leptin gene and its receptor are involved in obesity, further studies on the correlation between *lep* and *lepr* levels of expression and the evolution of adipogenesis in swine populations need to be conducted.

This study aims to investigate the gene expression levels of *lep* and *lepr* in the adipose tissue of different swine breeds to correlate their expressions with fat accumulation. For this, the expression levels in one breed characterized by the ability to accumulate fat (Mangalitsa) were compared to those of 5 specialized breeds that are used for meat production (Large White, Landrace, Duroc, LSP-345, and LSP-2000). Additionally, the *lepr* expression levels in different tissue types from Mangalitsa breed specimens were investigated.

MATERIAL AND METHODS

Biological material and sampling

The biological source was represented by obtaining samples from different tissues. The total RNA extracted from the adipose tissue and harvested from Mangalitsa, Duroc, Large White, and Belgian Landrace swine breeds and from LS-345 and LSP-2000 synthetic lines was used for the analysis of *lep* and *lepr* gene expression levels. Further, the total RNA isolated from the liver, spleen, kidney, heart, brain, and skeletal muscle tissues from Mangalitsa were used to assess *lepr* gene expression.

Total RNA extraction and reverse transcription reaction

Total RNA was extracted using the TriReagent kit (Sigma, Germany), and reverse

transcription reaction was performed using iScript™cDNA synthesis kit (BioRad, USA). The quality of total RNA extracted was estimated using Bioanalyzer 2100 (Agilent, Germany). Reverse transcription reaction was performed in 3 steps: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

Relative quantification of *lep* and *lepr* gene expressions and statistical analysis

The relative *lep* gene expression levels among Mangalitsa, Duroc, Large White, Belgian Landrace, LS-345, and LSP-2000 were quantified using real-time polymerase chain reaction (PCR) (Table 1). *gapdh* and *rpl32* genes were used as reference genes for data normalization. All experiments were performed in triplicate (N = 3).

Relative gene expression of *lepr* was also determined using the real-time PCR technique (Table 1). The expression was comparatively quantified i) in the adipose tissue derived from Mangalitsa, Duroc, Large White, Landrace, LS-345, and LSP-2000 and ii) between different tissues (liver, spleen, kidney, heart, brain, and skeletal muscle).

Table 1. Sequence of primers used for the amplification of target and reference genes.

Gene	Primer sequences
Leptin (*lep*)	F: 5'-TTGGCCCTATCTGTCCTACG-3' R: 5'-TTTCTGGAAGGCAGACTGGT-3'
Leptin receptor (*lepr*)	F: 5'-GTGAAGCCTGATCCACCATT-3' R: 5'-CCCCTCACCTGAACCTCATA-3'
Glyceraldehyde-phosphate dehydrogenase (*gapdh*)	F: 5'-GGGCATGAACCATGAGAAGT-3' R: 5'-GTCTTCTGGGTGGCAGTGAT-3'
Ribosomal protein 32 (*rpl32*)	F: 5'-TGCTCTCAGACCCCTTGTGAAG-3' R: 5'-TTTCCGCCAGTTCCGCTTA-3'

The PCR analyses were performed in a total volume of 25 µL by using iQ™SYBR®Green Supermix kit (BioRad) and IQCycler device (BioRad). The reactions consisted of 4 stages: an initial step of 5 min at 95°C, 45 amplification cycles of 30 s at 95°C, 30 s at 61°C, 25 s at 72°C, melting curve (1 cycle of 1 min at 95°C, 1 cycle of 1 min at 55°C, and 85 cycles of temperature increase over 0.5°C steps), and a final step at 20°C. All experiments were performed in triplicate and, for each breed, 3 tissue specimens were selected.

The two-tailed or unpaired *t*-test was used for statistical analysis. The P values less than 0.05 were considered to be statistically significant.

RESULTS

Assessment of relative gene expression of *lep* in different swine breeds

After the data were normalized to both reference genes, relative gene expression of *lep* was revealed, as shown in Figure 1. The highest levels of expression were found in the Mangalitsa breed, followed by those in the Belgian Landrace specimens, and the lowest were found in the remaining breeds analyzed in this study.

The gene expression levels were very significantly higher between Mangalitsa and Large White, Mangalitsa and LSP-2000, and Mangalitsa and LS-345 ($P < 0.001$) and significantly higher ($P < 0.01$) between Mangalitsa and Duroc and between Mangalitsa and Belgian

Landrace. The most important statistical differences were observed between *lep*-related expression between Mangalitsa and Large White breed (approximately 6 times higher expression for Mangalitsa breed), LSP-2000 (approximately 6.2 times higher expression for Mangalitsa breed), and LS-345 (approximately 6.5 times higher expression for Mangalitsa breed). Relative *lep* gene expression in Mangalitsa was found to be approximately 4 times higher than that of the Duroc breed, and that between Mangalitsa and Belgian Landrace was only 2.3 times higher (Figure 2).

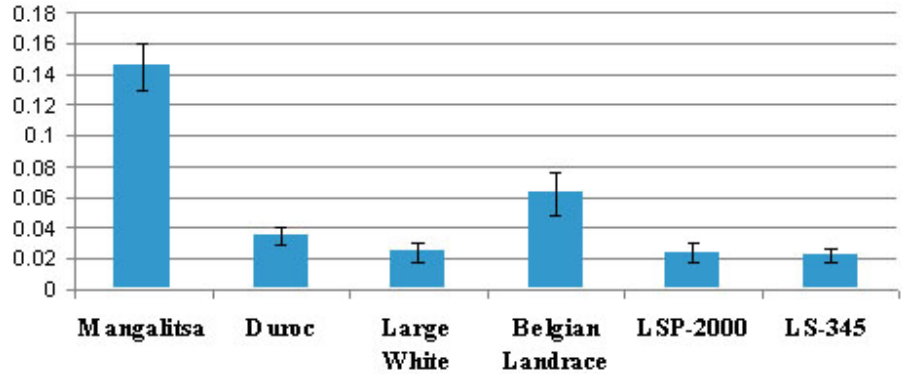

Figure 1. *lep* relative gene expression in different swine populations.

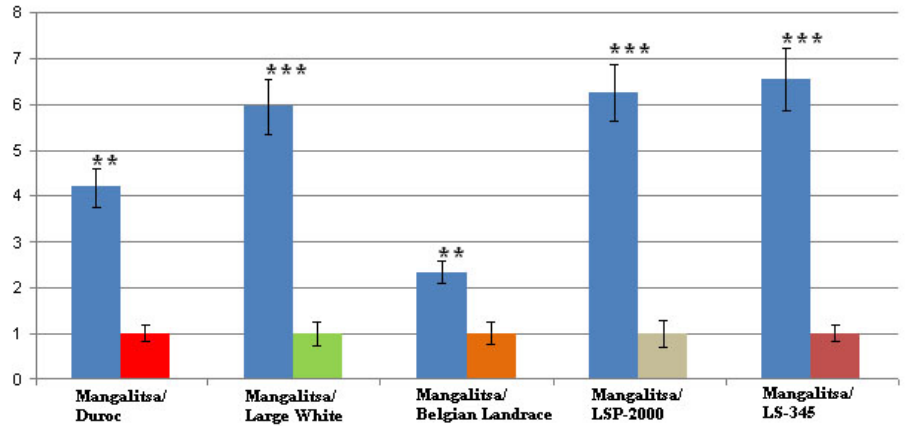

Figure 2. Comparison of *lep* relative gene expression levels between Mangalitsa and Duroc/Large White/Belgian Landrace/LSP-2000/LS-345 breeds (**P < 0.01, ***P < 0.001).

Assessment of relative *lepr* gene expression in different swine breeds

When the expression levels of *lepr* was analyzed after normalization of all samples to 2 reference genes, mean values and standard deviations obtained were plotted (Figure 3). The primers used in this study amplified a fragment corresponding to the extracellular domain, common to all forms of the *lepr*; thus, the relative quantification of the expression is available for all receptor isoforms. The lowest *lepr* expression was recorded for Belgian Landrace

breed, whereas gene expression levels found in Mangalitsa and Large White were even lower than those for Duroc breed or synthetic lines LSP-2000 and LS-345. The highest level of *lepr* expression was identified in LS-345.

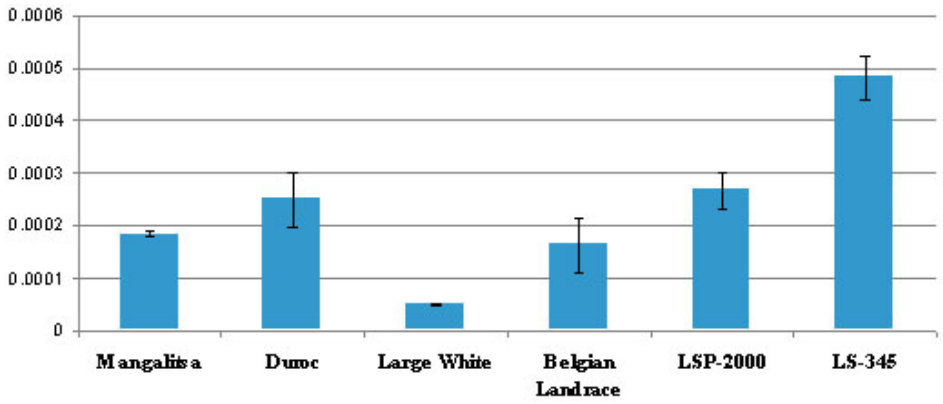

Figure 3. *lepr* relative gene expression in different swine populations.

Statistical comparison of relative *lepr* gene expression levels between Mangalitsa and other breeds was performed using two-tailed or unpaired *t*-test. Expression levels were not significant ($P > 0.05$) between Mangalitsa and Duroc, Large White, or LSP-2000, but were significant ($P < 0.05$) between Mangalitsa and Belgian Landrace; they were highly significant ($P < 0.01$) between Mangalitsa and the LSP-345 synthetic line (Figure 4).

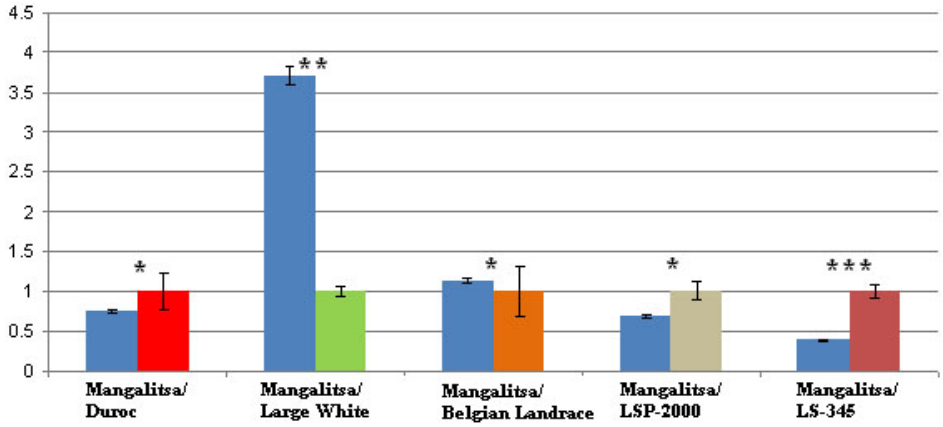

Figure 4. Comparison of *lepr* relative gene expression levels between Mangalitsa and Duroc/Large White/Belgian Landrace/LSP-2000/LS-345 (*$P > 0.05$; **$P < 0.05$; ***$P < 0.01$).

Statistical significant differences were found between Mangalitsa and Large White/LS-345 expression levels, while the expression levels were not significant among all the other breeds. Relative *lepr* expression was 3.7 times higher in Mangalitsa compared to that in Large White and 2.6 times lower than that in LS-345.

Comparative studies of relative *lepr* gene expression in different types of tissues from Mangalitsa breed

After all samples were normalized to the reference genes, mean values and standard deviation obtained for *lepr* gene expression in different tissues were plotted (Figure 5). The *lepr* gene was expressed in all tissues analyzed, but the lowest relative expression was found in the brain and skeletal muscle, while the highest expression was detected in the liver.

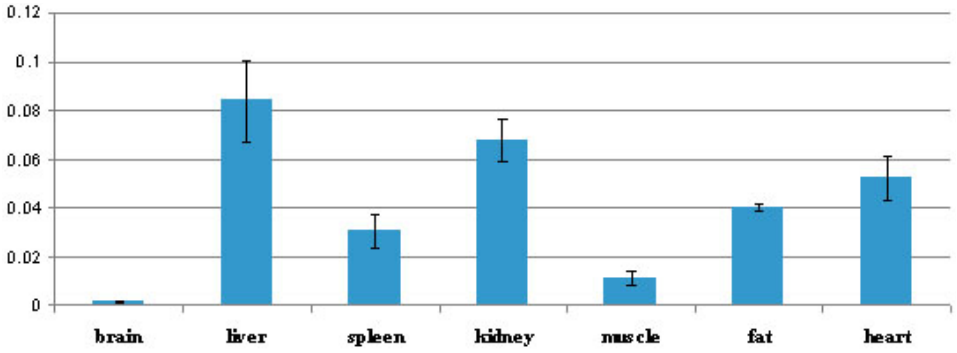

Figure 5. *lepr* relative gene expressions in different tissues from Mangalitsa breed.

DISCUSSION

Leptin is considered an important regulator of appetite, energy metabolism, body composition, and reproduction. Circulating leptin concentration was found to be correlated with body mass index and total body fat (Banks, 2004). According to Meier and Gressner (2004), when the energy produced in the body is equal to that consumed and when the total body weight is maintained constant, leptin levels reflect the amount of total body fat.

Previous studies have associated some polymorphisms found in the gene encoding the leptin receptor to some reproductive characteristics in swines (Chen et al., 2004) or to the concentration of circulating leptin (Kuehn et al., 2009). Apart from these findings, the leptin receptor has been thought to be indirectly related to obesity since it controls appetite.

The *lepr* gene is located on chromosome 6, in a region where a quantitative trait locus was detected. According to Uemoto et al. (2012), this quantitative trait locus is related to fat thickness or serum leptin. However, poor expression of leptin receptor in certain tissues was suggested to lead to leptin resistance, which is commonly associated with obesity. Studies performed in obese individuals have shown that these individuals exhibit insulin and leptin resistance, as well as elevated plasma levels of both hormones (Igel et al., 1996). Certain features such as excessive accumulation of body fat, the presence of increased amounts of intramuscular fat, and relatively low fertility in Mangalitsa breed might be associated with an increased leptin gene expression.

Our results suggest that a leptin resistance phenomenon occurred in the Mangalitsa breed. Thus, although relative *lep* expression is considerably higher in Mangalitsa specimens, these pigs are resistant to leptin and simultaneously show increased fat accumulation and reduced reproductive functions.

Results obtained in this study are consistent with those already reported in the literature. Ramsay et al. (1998) previously correlated the increase of leptin expression from the adipose tissue and the levels of circulating leptin with obesity in swine populations. Similarly, increase in circulating leptin was detected in obese human individuals (Klein et al., 2004). Therefore, despite the high levels of leptin, which should be responsible for a decrease in food and fat assimilation, obese individuals were observed to be also resistant to leptin. Research on leptin resistance suggested that the main sites of resistance were tissues with high metabolic activity, such as muscle tissues (Westerterp-Plantenga et al., 2001).

Decreased leptin receptor gene expression was found in the brain and muscle tissues of the Mangalitsa breed. The hypothalamus appears to be the main area of leptin action, since there is a high density of receptors for leptin, especially in the areas responsible for controlling appetite, reproduction, and growth (Tartaglia et al., 1995; Dyer et al., 1997). Our results are consistent with those obtained by Lin et al. (2000), who revealed low levels of *lepr* expression in the cerebral cortex.

The expression of *lepr* was found to be higher in the renal tissue. The role and function of leptin at this level have not yet been completely elucidated. However, leptin was thought to inhibit α1-hydroxylase expression in *ob/ob* mice (Matsunuma and Horiuchi, 2007).

In the liver, *lepr* expression was found to be higher than that found in the adipose tissue. Gallardo et al. (2007) reported an interesting effect of leptin on hepatic and adipose tissues. They suggested that leptin regulates *de novo* lipogenesis by increasing lipolysis and fatty acid release from adipose tissue, along with fatty acids acquisition and oxidation in the liver tissue. This leptin activity was strongly correlated with its action on the central nervous system.

The muscle tissue is considered to be the main site of leptin resistance (Westerterp-Plantenga et al., 2001), and our results revealed low levels of *lepr* gene expression in this type of tissue. According to Fuentes et al. (2010), leptin receptors and signaling in the skeletal muscle are reduced in obese humans. Our results are in agreement with the findings of their study, since the lowest levels of relative *lepr* gene expression in the Mangalitsa breed, which is characterized by high levels of intramuscular fat, were found in the brain and skeletal muscle tissues.

The results obtained in this study showed an increased expression of leptin gene in the Mangalitsa breed compared to that in the other breeds of swines specialized in meat production. Leptin receptor gene expression was lower in the Mangalitsa breed than in the Duroc, Large White, LS-345, and LSP-2000 breeds.

Additionally, the comparative analysis of *lepr* expression in different tissue types from Mangalitsa revealed an increased expression in the liver, kidney, spleen, adipose tissue, or heart and a very low level of expression in the central nervous system and skeletal muscles. These results might suggest that the Mangalitsa breed exhibits resistance to leptin, which can be associated with a very strong tendency for fat accumulation and a below-average prolificacy. Studies regarding leptin resistance in Mangalitsa breed should be addressed in further studies, in order to understand obesity and its related diseases in humans.

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