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Searching for genes controlling fatness traits in pigs – a review*

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Fat content of carcass is an important multigenic trait in pig breeding. There are reports indicating several chromosomes, e.g. 1, 2, 4, 5, 6, 7, 12, 13, 14 and 18 which possibly harbour QTLs for fatness traits. Among QTL candidate genes there are leptin (*LEP*) and its receptor (*LEPR*), both playing essential role in food intake and energy balance. Moreover, expression level and polymorphism of the adipocyte specific transcription factors, such as CREB (cAMP response element binding protein) or C/EBP (CCAAT/enhancer-binding protein) may also cause phenotypic variation of the fatness traits. Some of the candidate QTLs, as *LEP*, *LEPR*, *C/EBP* and additionally *H-FABP* (fatty acid binding protein gene) and *RYR1*, are localized on chromosome 6. It is foreseen that searching for polymorphisms of the chosen genes may reveal association between a genotype and phenotypic variation of selected fatness traits. However, the studies are complex and require analysis of numerous genes. Cited are 71 references.

KEY WORDS: fatness traits / leptin / leptin receptor / marker genome map / QTL / pig

An advanced marker genome map facilitates the detection of genes controlling productive traits by genome scanning. The efficiency of this approach depends on the density of the marker genome map. In the porcine genome map there are already 2690 *loci*, out of which 928 are designated as genes (ARKdb, Pig data base, Roslin Institute, http://www.thearkdb.org). Since the haploid number of chromosomes (n) in the pig is

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19, it may be assumed that marker density of the map is sufficient to conduct preliminary searching for any gene of interest which controls a trait with a known phenotypic variation. In such studies information on genome maps of other species is also helpful. Chromosome homology, revealed by the so-called comparative chromosome painting, was presented for the pig and human, as well as cattle and sheep chromosomes [Fronicke *et al*. 1996, Goureau *et al*. 1996, Schmitz *et al*. 1998, Fronicke and Wienberg 2001]. Also detailed comparisons of gene order in selected chromosomes of the pig and human were made. For instance, a high homology of the gene order between human chromosome 12 and pig chromosome 5 [Goureau *et al*. 2001], or human chromosome 21 and pig chromosome 13 [Tuggle *et al*. 2001] have recently been reported. On the other hand, it was shown that human chromosome 3 and pig chromosome 13 show a complete synteny conservation, but extensive differences in the gene order [Sun *et al*. 1999]. The above examples indicate that information on homology between chromosome fragments in the human and pig genomes not always reflects homology of gene order.

There are several approaches which can be applied in the studies on the detection of quantitative trait *loci* (QTLs): (1) segregation analysis of markers in two- or three-generation reference families in which phenotype variation of traits is recorded along with the segregation of alleles representing a large number of evenly spread marker *loci*; (2) molecular analysis of *a priori* selected genes which potentially may play a major role in the genetic control of a trait; (3) analysis of tissue-derived cDNAs (muscle, fat, etc.) and (4) the so-called *in silico* mapping (mapping with the use of available electronic databases). In the recent years the two latter approaches have become very powerful and promising. Davoli *et al*. [2002] analysed cDNAs developed from porcine skeletal muscles, and mapped 107 DNA fragments onto the pig chromosomes, using the somatic cell hybridization approach. Among them at least one gene, namely *ADFP* (adipocyte differentiation-related protein) is strictly related to the function of adipocytes. On the other hand, *in silico* mapping was applied by Farber and Medrano [2003]. The authors compared flanking sequences of 1570 porcine microsatellites with EST (expressed sequence tags) deposited in GenBank databases and found 44 sequences which had at least one match to the human genomic sequence or ESTs and could be localized in the pig genome. The EST porcine map, based on human and porcine ESTs, has recently been developed within the European GENETPIG project [Karsenty *et al* 2003]. The authors reported the mapping of 630 ESTs in the pig genome – http://www.infobiogen.fr/services/Genetpig/.

Genome scans to identify chromosome regions with genes controlling fatness traits

Searching for chromosomal regions carrying QTLs for traits of economic importance, with the use of marker genome maps, has become a common strategy applied in major livestock species, including the pig. In the very first publication devoted to this topic Andersson *et al*. [1994] analysed the segregation of 105 genetic markers along

with the analysis of phenotypic variation of some production traits in the F_2 generation of a three-generation reference family, derived from Large White × wild boar crosses. In further studies, carried out by other authors, different resource families were also considered, e.g. Large White × Złotnicka Spotted [Kurył *et al*. 1998], Berkshire × Yorkshire [Malek *et al*. 2000a, 2000b], Meishan x Large White [Milan *et al*. 2002], and Iberian Goudyerbas line × Landrace [Ovilo *et al*. 2002]. It is obvious that the analysis of genetically different resource families may lead to inconsistent results for the same traits, due to breed-specific gene pools and interactions between them. Results obtained in the nineties, with regard to a wide range of production traits, were reviewed by Kurył [2000] and Schwerin [2001]. In the recent years new data were reported concerning the localization of the QTLs for fat deposition i pigs.

An extended study on the detection of QTLs for carcass composition was reported by Milan *et al*. [2002]. The authors identified several chromosome regions which may carry QTLs for fatness traits; among them QTLs for backfat weight were detected on chromosome 1, 2, 4, 7, 11 and X. In that study the intramuscular fat content was not included, however considered were leanness traits. Among them the ECLC indicator (estimated carcass lean content based of the relation between ham, loin, backfat, leaf fat and belly) was considered. QTLs for this trait were detected on the same chromosomes – 1, 2, 7 and X. Suggestions concerning chromosomes 2 and 7 were earlier presented by Rattink *et al*. [2000] who carried out a detailed study of chromosomes 2, 4 and 7 and found evidence for the backfat thickness QTL, but not for intramuscular fat content, on chromosome 2 and 7. The X chromosome as a carrier of QTLs for backfat and intramuscular fat content was suggested also by Harlizius *et al*. [2000].

Scanning of the porcine genome, with the use of 125 microsatellite markers covering the entire genome, revealed evidence for QTLs influencing backfat thickness (measured at different points) on chromosomes 1, 4, 5, 6, 7, 12, 13, 14 and 18 [Malek *et al*. 2001a]. On the other hand, QTLs for intramuscular fat were found on chromosomes 1, 8 and 10. In addition, QTLs for the total lipid per cent and cholesterol concentration were indicated on chromosomes 1 and 18, respectively [Malek *et al*. 2001b].

Interesting results were reported for chromosome 6 by Ovilo *et al*. [2000] who indicated that QTLs for intramuscular fat and backfat thickness reside on chromosome 6, between S0228 and Sw1881 microsatellite markers. A similar suggestion was presented by Grindflek *et al*. [2001] who showed that the QTL for intramuscular fat is flanked by SW1823 and S0228 microsatellite markers. The polymorphism effect of two genes, namely heart fatty acid binding protein (*H-FABP*) and leptin receptor (*LEPR*), which are located in the region of interest, was tested in further studies carried out by Ovilo *et al*. [2002]. Both polymorphisms were caused by silent nucleotide substitutions in intron 2 and 4, respectively. The *H-FABP* gene appeared to be associated with intramuscular fat content, but not with backfat thickness, while the *LEPR* gene with both intramuscular fat content and backfat thickness. It is important to emphasize that both polymorphisms were not casual mutations for these effects.

The studies mentioned revealed that QTLs for backfat may reside on numerous

pig chromosomes. On the other hand, certain chromosomes (1, 4, 6, 7 and X) were detected in independent studies, carried out on different reference families. Moreover, QTLs for the intramuscular fat content were also found on chromosomes 1, 6 and X indicating a genetic correlation between both fatness traits. Thus, these chromosomes may be recommended for further detailed studies. Kurył [2000] reviewed results of similar studies carried out in the years 1994-1999 and also showed numerous chromosomes with evidence for backfat (nos. 1, 2, 4, 5, 6, 7, 8, 9, 10, 13, 15 and X) and/or intramuscular fat (nos. 2, 4, 6, 7) QTLs. Considering all the results mentioned it can be concluded that the segregation of a single gene with a really large effect on both traits in all studied reference families, is rather unlikely. It seems more probable that in different populations may segregate specific alleles with rather moderate effect. On the other hand, these studies showed some chromosomes (nos. 1, 4, 6, 7 and X) which should be carefully studied.

Genomic studies on mammalian species revealed several genes, which may play a key role in the control of fatness traits. The localization of these genes (Tab. 1) indicates that at least some of them reside on chromosomes indicated as carriers of QTLs for fatness traits. The effect of some genes – leptin (*LEP*), leptin receptor (*LEPR*), heart and adipocyte fatty acid-binding proteins (*H-FABP* and *A-FADP*, respectively) – was already studied in the pig. The association of *H-FABP* and *A-FABP* genes polymorphisms with intramuscular fat and backfat content was suggested by Gerbens *et al*. [2000]. There are also other genes, which can also be considered as candidate QTLs, since they are involved in adipocytogenesis, e.g. those of *CEBPα* and *CREB* transcription factors and adipose differentiation-related protein (*ADFP*). Our studies were focused on four *a priori* selected genes which are biologically related to energy balance (*LEP* and *LEPR*) and adipocytogenesis (*C/EBPα* and *CREB*).

Table 1. Selected genes which may be considered as candidate QTLs for fatness traits in pigs

Locus	Gene	Chromosome No.
LEP	leptin	18
LEPR	leptin receptor	6
H-FABP	heart fatty acid-binding protein	6
A-FABP	adipocyte fatty acid-binding protein	4
ADFP	adipose differentiation-related protein	1
C/EBPβ	CCAAT/enhancer-binding protein	2* or 6*
C/EBPδ	as above	17*
C/EBPγ	as above	?
CREB	cyclic AMP response element-binding protein	3* or 15*
PPARδ	peroxisome proliferator-activated receptor	13*

*Localization anticipated from the human-pig comparative chromosome painting [Frönicke *et al*. 1996].

Leptin and leptin receptor genes – comparative studies

The leptin gene (*LEP*) was mapped in the pig chromosome 18q13-q21 [Neuenschwander *et al*. 1996]. From the studies on the human and mouse genomes it is known that *LEP* gene is composed of three exons and two introns. Its structure in the pig genome is rather poorly described – until now the DNA sequence, comprising of a part of intron 1, exon 2, intron 2 and a part of exon 3, was deposited with the GenBank (Accession number U66254) – Bidwell *et al*. [1997]. Both the structure of the leptin gene and the mRNA processing were already described in a review article by Madeja *et al*. [2002]. A comparative study of the known DNA sequences showed a high homology between the porcine and human (93%) or porcine and mouse (92%) sequences. This observation indicates that the structural part (exons and introns) of the leptin gene is evolutionary highly conservative.

There are no data available concerning porcine 5'- and 3'-flanking DNA sequences of the *LEP* gene. A comparative analysis of human, cattle and mouse 5'-flanking sequences reveals quite a high homology (Fig. 1). Between the transcription initiation site and the TATA box a sequence Sp1 is present, important for the protection of the DNA helix against DNAse I. This motif occurs also in other sites. Upstream from the TATA box there are several sequences recognized by C/EBP (CCAAT/enhancer-binding protein) and CREB (cAMP response element-binding protein) transcription

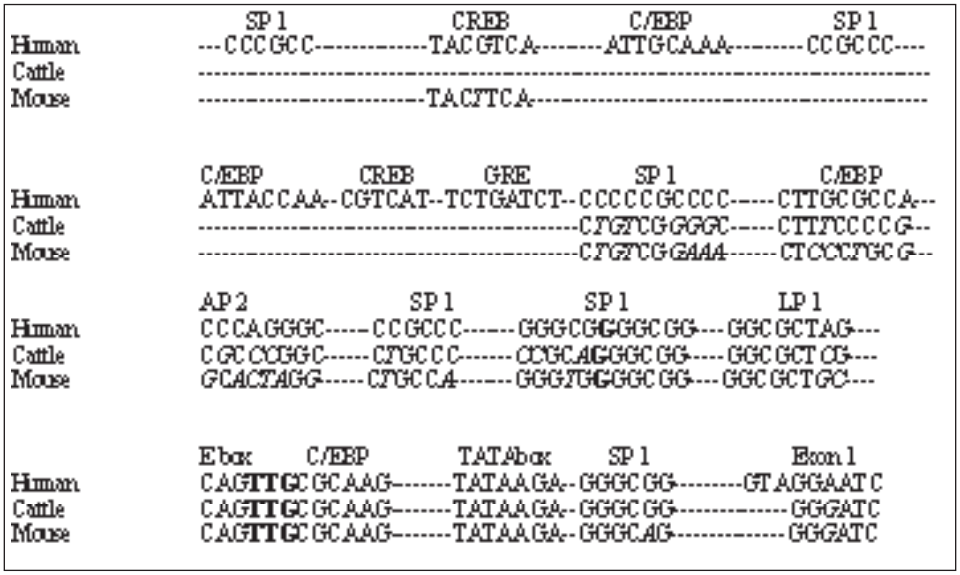

Fig. 1. Comparison of human, bovine and mouse leptin gene promoter sequences. Transcription factor-binding sites are shown in a letter code. Fragments of the consensus sequences which are common for the two transcription sites are bolded. Regions which are non-conservative are shown in italics.

factors. The crucial role of the C/EBPá for the transcriptional activation of the leptin gene was experimentally confirmed both in man and mouse [Hwang *et al*. 1996, Miller *et al*. 1996]. There are also other regulatory sequences: GRE (glucorticoid response element), E box and AP2 binding site [Gong *et al*. 1996, Isse *et al*. 1995, Mason *et al*. 1998). However, in the mouse and cattle genomes some of the above sequences were not identified in spite of their presence in the human leptin gene. It can be anticipated that also in the pig genome not all sequences found in man are present.

Knowledge on the porcine leptin receptor gene (*LEPR*) mapped onto chromosome 6q33-q35 is also very limited. Gene structure and the presence of different Lepr isoforms was reviewed recently by Madeja *et al*. [2002]. Only a small part of the porcine Lepr gene DNA sequence, comprising exons 3, 4 and 5, was described [Ruiz-Cortez *et al*. 2000]. For the human Lepr the cDNA sequence is known, representing all twenty exons [Tartaglia *et al*. 1995]. Unfortunately, there are no data concerning the 5'-flanking sequence of this gene. Surprisingly, a comparison between human, mouse and rat 5'-sequences, based on available genomic DNA sequences (GenBank Accession numbers: human – NT 004636, mouse – NT 039264, rat – NW 043856), did not reveal homology. We were not able to find the TATA box in the 5'-flanking human DNA sequence. It is known that not all promoters contain this sequence, thus it may potentially also concern the *LEPR* gene.

Phenotypic effects of *LEP* and *LEPR* polymorphisms

Phenotypic effects of point mutations in the *LEP* and *LEPR* genes were first described in the mice [Zhang *et al*. 1994, Chen *et al*. 1996]. Also in human there are some reports on this association. For example, polymorphism G2548A in the 5'-flanking region was associated with extreme obesity in women and the G allele more frequently found in overweight individuals [Mammes *et al*. 2000].

In both genes several polymorphic sites were identified in the pig. In the *LEP* gene the first polymorphism found in the pig was a silent substitution C→T at position 3469 (exon 3) which is recognizable by the *Hinf*I restriction enzyme [Stratil *et al*. 1997]. In the same exon another silent substitution G3714T identified by the *Pst*I restriction enzyme was described by Jiang and Gibson [1999] who also found two other substitutions in intron 1 (C867T) and intron 2 (A1112G), detected by the *Taq*I restriction endonuclease. Kennes *et al*. [2001] described substitution A2845T in intron 2, recognized by the *XbaI* restriction enzyme. A lack of mutations causing the alteration of the amino acid suggests that its sequence is highly conservative. Thus, it seems to be rather unlikely that in modern pig breeds a causative mutation modifying the leptin polypeptide may segregate with a moderate or high frequency. Of course isolated cases of such mutations may appear.

In spite of the fact that all the identified mutations are silent substitutions, there were several attempts to analyse the association between the polymorphisms and growth and carcass traits. Jiang and Gibson [1999] reported a relationship between fat depth

and the T3469C polymorphism. In a group of 32 Large White pigs a higher frequency of the *C* allele was correlated with the lower level of the investigated trait. However, in the second group of 40 this result was not confirmed. The same polymorphism was found to be associated with growth traits (daily live weight gain to weaning, weight at weaning, weight of right carcass) and the genotype *TT* appeared to be more advantegous than the *TC* genotype [Krěnkowa *et al*. 1999]. Different results were presented by Kulig *et al*. [2001], who claimed that pigs of the genotype *TC*, in terms of mean daily live weight gain and lean meat content of carcass, are better than those of the *TT* genotype. In both studies the *CC* genotype was not included due to its low frequency. On the contrary, Kurył *et al*. [2003] analysing 249 unrelated animals revealed that the *TT* genotype was associated with lower fat weight and lower fat content of ham. A comparison of these results allows to coclude that polymorphism of the leptin gene may be associated with different pig production traits. But this is not a causative mutation, and the observed phenotypic variation may be caused by other genes, potentially linked with the *LEP locus*. In different populations (breeds) one can anticipate differences in gene pools and thus observed phenotypic effects are not consistent. On the other hand, inconsistency of the observed effects may depend on the statistical method applied and the number of animals examined. In the above mentioned studies the total number of genotyped animals was 95 Large White × Landrace crosses [Krěnkova *et al*. 1999], 131 Polish Landrace pigs [Kulig *et al*. 2001] and 249 pigs of various breeds and lines [Kurył *et al*. 2003]

In the leptin receptor gene four polymorphisms were described. Two are intronic substitutions, recognized by the *Hpa*II and *Rsa*I restriction enzymes [Stratil *et al*. 1998]. Localization of the other two within the gene remains unknown. One of them is detected by *Hinf*I [Vincent *et al*. 1997] and the other by DGGE technique [Kopečny *et al*. 1997].

Transcription factors involved in adipocyte differentiation

The process of adipocyte differentiation is a series of chronological events. First a multipotent stem cell precursor gives rise to a mesenchymal precursor cell, which can differentiate into preadipocytes. After the growth arrest of proliferating preadipocytes cells undergo at least one round of DNA replication and cell doubling. This process of clonal expansion is ceased with the beginning of the PPARγ and C/EBPα expression. Several transcription factors act cooperatively to trigger the differentiation process, which finally leads to the creation of mature adipocytes [Rosen *et al*. 2000, Gregoire *et al*. 1998].

Absolutely required for adipogenesis, both *in vitro* and *in vivo*, is the peroxisome proliferator-activated receptor γ – **PPARγ** – Rosen *et al*. [2002]. The PPARα belongs to the superfamily of the steroid hormone nuclear receptors and forms heterodimers with the retinoid X receptor (RXR) – Juge-Aubry *et al*. [1995]. The DNA response element involves 2 half-sites composed of the hexameric sequences AGGTCA sepa-

rated by only one nucleotide [Clarke *et al*. 1999]. The structural organization of this superfamily member is similar and comprises an N-terminal domain, a DNA binding domain including two zinc fingers, and also at the carboxyl terminus a dimerization and ligand binding domain [Rosen and Spiegelman 2001].

There are two main isoforms of PPARγ: PPARγ1 and PPARγ2 [Tontonoz *et al*. 1994], and additionally the third type – PPARγ3 – the protein indistinguishable from the PPARγ1 [Fajas *et al*. 1998]. The human *PPARγ* gene spans more than 100 kb and is organized in six common exons (all types of PPARă contain this region). In addition to the common exons, PPARγ1 comprises exon A1 and A2, PPARγ2 exon B and PPARγ3 exon A2. The amino acid sequences of PPARγ1 and PPARγ3 are identical, since exons A1 and A2 are untranslated. Exon B, which is located between exon A2 and exon 1, codes for additional 28 amino acids of PPARγ2 [Fajas *et al*. 1997]. The amino acid sequence comparison indicated a 97% identity between the human and mouse PPARγ2 [Mukherjee *et al*. 1997]. PPARγ2 seems to play a key role in the initiation of adipogenesis [Saladin *et al*. 1999].

Three variants of the human *PPARγ* gene were identified: $Pro^{12}Ala$, CAC478CAT and $Pro^{115}Gln$. Individuals with $Ala^{12}Ala$ and CAT478CAT genotypes were severely obese and had increased fat mass [Valve *et al*. 1999].

The family of transcription factors – the CCAAT/enhancer-binding proteins (CEBPs), plays a key role in inducing preadipocyte differentiation and in modulating gene expression in adipocytes. The CEBP family comprises six genes: α, β, γ, δ, ε and ζ which probably arose by duplication events. CEBPβ and CEBPδ are active early in adipogenesis and are important for directing the differentiation process [Darlington *et al*. 1998, Lekstrom-Himes and Xanthopoulos 1998].

CEBPα is sufficient for adipocyte differentiation. Its antimitotic activity, cooperatively with PPARγ, terminates clonal expansion [Rosen *et al*. 2000]. The intronless gene codes for two isoforms of C/EBPα – the full length 42-kDa C/EBPα and 32-kDa product, which is a result of the initiation of translation at the third in-frame AUG codon [Lin *et al*. 1993, Ossipow *et al*. 1993]. C/EBPα activates the promoters of several adipocyte genes, including PEPCK, leptin, and the insulin receptor [Gregoire *et al*. 1998]. Surprisingly, C/EBPα and PPARγ can functionally antagonize on the leptin promoter [Hollenberg *et al*. 1997].

A protein of this family member consists of a carboxyl terminal leucine zipper, a DNA binding domain and a nuclear localization signal. The remaining N-terminal amino acids (transactivation domain) contain insuline-responsive sites of phosphorylation [Lekstrom-Himes and Xanthopoulos 1998]. This transactivation domain consists mainly of conserved elements – CR1, CR2, CR3, and CR4 [Erickson *et al*. 2001].

The cyclic AMP response element-binding protein – **CREB** – has been shown to be sufficient for adipogenesis *in vitro* [Reusch *et al*. 2000]. CREB stimulates the transcription of proteins containing the cAMP response element (CRE). The CRE is an 8 bp palindrome, TGACGTCA, containing two inverted CGTCA half-sites [Quinn 2002]. The CREB protein consists of the acidic region with a cluster of potential

phosphorylation sites [Gonzalez *et al*. 1989], and the C-terminal positively charged basic region – the leucine zipper [Hoeffler *et al*. 1990]. CREB stimulates transcription through a constitutive activation domain (CAD) and a kinase-inducible domain (KID) – Kim *et al*. [2000].

The CREB gene is organized in 11 exons and 10 introns generating multiple mRNA isoforms [Ruppert *et al*. 1992]. The two forms of the CREB protein – one of 341 amino acids termed CREB-A and the other of 327 amino acids called CREB-B, are encoded by alternative transcripts of a single gene [Berkowitz and Gilman 1990]. A comparison of the human and rat CREB cDNA shows a high conservation between the nucleotide and amino acid sequences - 96 and 99%, respectively [Hoeffler *et al*. 1990].

Studies of gene expression during adipocytogenesis revealed quite different expression profiles *in vivo* and *in vitro*. It indicates that this process is likely more complex than previously suggested [Soukas *et al*. 2001].

Conclusion

The identification of QTLs controlling fatness traits seems to be a difficult task. Nine years after the first publication of Andersson *et al*. [1994] on genome scan with the use of polymorphic markers, the achieved progress is rather modest. It is partly caused by decreasing genetic variation (limited gene pools) in modern pig breeds due to selection for meat deposition traits. On the other hand, existing differences between gene pools, characteristic for specific breeds, make it difficult to directly extrapolate results obtained for one breed (or reference family) to another one. Genome scans have also brought surprising results that chromosome 18, on which the leptin gene resides, was rarely detected as a carrier of QTLs for fatness traits. It is anticipated that a new generation of the genome maps – the EST map – should bring new data, which will facilitate the identification of other genes controlling fatness traits. A high homology of the structural parts of *LEP* and *LEPR* genes among mammals shows that the main target of further studies should be the analysis of the regulatory sequences and variation of gene expression profiles.

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Poszukiwanie genów kontrolujących cechy otłuszczenia świń – artykuł przeglądowy

S t r e s z c z e n i e

Odkładanie tkanki tłuszczowej jest ważną cechą produkcyjną świń, warunkowaną przez wiele genów. Istnieją doniesienia wskazujące, że w różnych chromosomach (np. 1, 2, 4, 5, 6, 7, 12, 13, 14 i 18) mogą występować geny o dużym efekcie działania (QTL) odpowiadające za zmienność fenotypową tych cech. Genami kandydującymi do roli QTL są m. in. gen leptyny (*LEP*) i receptora leptyny (*LEPR*). Ich białkowe produkty odgrywają istotną rolę w kontroli pobierania pokarmu i utrzymywania równowagi energetycznej. Zmienność cech otłuszczenia może być m.in. wywołana zróżnicowanym poziomem ekspresji i polimorfizmu specyficznych dla adipocytów czynników transkrypcyjnych, takich jak CREB (*cAMP response element-binding protein*) czy C/EBP (*CCAAT/enhancer-binding protein*). Kilka potencjalnych QTL, takich jak *LEP*, *LEPR*, *C/EBP*, a także *H-FABP* i *RYR1*, zlokalizowanych jest w chromosomie 6. Przewiduje się, że poszukiwanie polimorfizmu wybranych genów może wykryć powiązania między genotypem i obserwowaną zmiennością cech otłuszczenia. Podkreślić jednak należy, że badania takie są złożone i wymagają analizy dużej liczby genów.

Powołano się na 71 pozycji piśmiennictwa.