



Cloning of *OLR1* Gene in Pig Adipose Tissue and Preliminary Study on Its Lipid-accumulating Effect

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ABSTRACT : In this study we cloned and characterized a novel lipid-accumulating gene, the oxidized low-density lipoprotein receptor 1 (*OLR1*), which is associated with lipogenesis. We analyzed the gene structure and detected the mRNA transcriptional expression levels in pig adipose tissues at different months of age (MA) and in different economic types (lean type and obese type) using real-time fluorescence quantitative PCR. *OLR1* expression profile in different tissues of pig was analyzed. Finally, we studied the correlation between *OLR1* and lipid metabolism related genes including peroxisome proliferator-activated receptor γ 2 (*PPAR γ 2*), fatty acid synthetase (*FAS*), triacylglycerol hydrolase (*TGH*), CAAT/enhancer binding protein α (*C/EBP α*) and sterol regulatory element binding protein-1c (*SREBP-1c*). Results indicated that the *OLR1* gene of the pig exhibited the highest homology with the cattle (84%), and the lowest with the mouse (27%). The signal peptide located from amino acid 38 to 60 and the domain from amino acid 144 to 256 were shared by the C-type lectin family. The expression level of *OLR1* in pig lung was exceedingly higher than other tested tissues ($p < 0.01$). In pig adipose tissue, the expression level of *OLR1* mRNA increased significantly with growth ($p < 0.01$). The expression level of *OLR1* mRNA in obese-type pigs was significantly higher than that of lean-type pigs of the same monthly age ($p < 0.05$). In adipose tissue, the expression of *OLR1* correlated with *PPAR γ 2*, *FAS* and *SREBP-1c*, but not *TGH* or *C/EBP α* . In conclusion, *OLR1* was highly associated with fat deposition and its transcription, as suggested by high correlations, was possibly regulated by *PPAR γ 2* and *SREBP-1c*.
(Key Words : *OLR1*, Pig, Lipid Metabolism, Adipose Tissue)

INTRODUCTION

Obesity has become an important issue in recent years and can be viewed as a state of body lipid-related disorders, including type 2 diabetes and atherosclerosis, that are related to excessive adipose tissue deposition and adipocyte hypertrophy (Larsen et al., 2003; Wang et al., 2008). It is widely accepted that oxidized low-density lipoprotein receptor 1 (*OLR1*) plays an important role in the process of cardiovascular diseases and shows binding activity to multiple ligands indicating that *OLR1* has complicated biological functions (Chen and Du, 2007). In mouse adipose tissue, the overexpression of *OLR1* can elevate the cholesterol content and this process is accompanied by an increase uptake of free fatty acid (FFA) and an accumulation of triglyceride (Chui et al., 2005), which demonstrates that *OLR1* has a close relationship with the storage of triglyceride and the generation of obesity.

Because of the rapid lipid deposition, the pig is

considered as a good model to study lipid metabolism. From this perspective, the local Chinese obesity-type pig is especially good material for many genes in adipocytes. Besides, there is a high homology among the pig, human and rodent, so the outcomes of our research can give a reference to humans. The current study aimed to clone the *OLR1* gene complete CDS in adipose tissue, analyze *OLR1* nucleotide and protein structure characteristics, and subsequently study *OLR1* expression in different tissues, different months of age (MA) and different economic types of pig adipose tissue. Additionally, investigation of the correlation between *OLR1* and other lipid metabolism-related factors was also carried out to appraise *OLR1* lipid-accumulation effects in adipose tissue. The data obtained in this research should be a guide for lean-type pig breeding and could also be a reference for the related diseases caused by lipid metabolism disequilibrium such as obesity and type 2 diabetes.

MATERIALS AND METHODS

Tissue materials preparations

Large White (LW) pigs at 2, 5 and 10 MA and

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Guanzhong Black (GB) pigs at 5 MA were obtained from the animal farm of Northwest A&F University. For each chosen age, 5 pigs were obtained for repeated experiments. After slaughter, samples from visceral adipose tissue, subcutaneous adipose tissue, muscle, heart, liver, spleen, lung and kidney were surgically collected and frozen in liquid nitrogen.

Reagents

TRIpure Reagent was purchased from Beijing BIOTEKE Biotechnology Company (Beijing) and RevertAid™ First Strand cDNA Synthesis Kit was from FERMENTAS (MBI)(Shanghai). *Taq* enzyme, Premix Ex Taq™ Hot start Version, SYBR Green and pMD18-T vector were from TaKaRa Company (Japan). DL2000Marker and plasmid trace-extraction kits were from Beijing TIANGEN Biochemistry Company (Beijing). *E. coli* DH 5 α was available in our own laboratory. Gel DNA purification kit was from Anhui UGENE Biotechnology Company (Hefei).

Primer design

According to the gene sequences of *ORL1* from related species published in GenBank, we obtained relevant gene assemblage sequences by the EST method using DNASTAR software, and then the relevant gene primers were designed by Primer5.0 software. The primers for *PPAR γ 2*, *FAS*, *TGH*, *C/EBP α* , *SREBP-1c* and β -actin were directly designed from relevant gene CDS in the pig using Primer5.0 software. Finally, the primer sequences were sent to Shanghai Invitrogen Biotechnology Company for primer synthesis (Table 1).

RNA extraction and first strand cDNA synthesis

RNA extraction followed TRIpure Reagent instructions and products were analyzed by 1% agarose gel electrophoresis. First strand cDNA was synthesized using Fermentas RevertAid™ First Strand cDNA Synthesis Kit: DEPC treated water 6 μ l, total RNA 5 μ l, 0.2 μ g/ μ l Random hexamers primer 1 μ l, 70°C for 5 min, after fast cooling add 4 μ l Buffer (5 μ l \times), 2 μ l dNTP Mixture(10 mmol/L), 1 μ l RNase inhibitor (20 u/ μ l), 25°C for 5min, 1 μ l reverse transcription enzyme (200 u/ μ l), now the total reaction volume was 20 μ l. Then 25°C for 10min, 42°C for 60 min, 70°C for 10 min, and stored at -40°C.

PCR amplification

PCR reaction system: the total reaction volume was 25 μ l, including 14.3 μ l double distilled water, 2.5 μ l 10 \times PCR buffer (Mg²⁺ plus), 2.5 μ l dNTP Mix (2.5 mmol/L), 1 μ l PrimerI (10 μ mol/L), 1 μ l PrimerII (10 μ mol/L), 0.2 μ l *Taq* enzyme (0.5 U/ μ l) and 1 μ l cDNA template. The amplification products were obtained by My Cycler™ Thermal Cycler (BIO-RAD) and the complete reaction conditions and parameters concerned with relative genes of PCR are listed in Table 1. The quantitative PCR reaction conditions were: 95°C for 10 s \rightarrow (95°C for 5 s \rightarrow 60°C for 30 s)*40 cycles \rightarrow 95°C for 15 s \rightarrow 60°C for 30 s \rightarrow 95°C for 15 s (extracted the amplification products).

Cloning of pig *ORL1* from adipose tissue

According to the instructions from the normal agarose gel DNA recovery kit, the specific band of GB pig *ORL1* gene was recovered from 1.5% normal agarose gel, the

Table 1. PCR parameters for relevant genes

| Gene | Primer sequences (5' \rightarrow 3') | Tm (°C) | Cycles | Length of amplified fragments |
|---------------------------------|--|---------|--------|-------------------------------|
| <i>ORL1</i> (complete CDS) | F: TGCTTG TAGAAGTCCTTGGCTG R: AGTTGTCAGGGCTTTCATGGT | 56 | 30 | 1,080 |
| <i>ORL1</i> (partial CDS) | F: TCTGGAGAAAGCGGCAAACT R: AAAGTGGGAATGGGCGATGGT | 56 | 30 | 200 |
| <i>PPARγ2</i> | F: ACCACTCGCATTCTTTGAC R: CCACAGACTCGGCACTCAAT | 52.1 | 32 | 261 |
| <i>FAS</i> | F: AGTGTCACCAACAAGCG R: GATGCCGTCAGGTTTCAG | 55.9 | 30 | 280 |
| <i>TGH</i> | F: CTTGGCTCCTTGAGATTTG R: AGTTGGCAATGTTGCTCTG | 53.3 | 32 | 455 |
| <i>C/EBPα</i> | F: GGTGGACAAGAAGCAACGA R: GATCTGGAGACCCGAAACCA | 52 | 32 | 320 |
| <i>SREBP-1c</i> | F: CTGGAGACATCGCAAACAAGC R: ATGGTAGACAACAGCCGCATC | 59.7 | 32 | 277 |
| β -actin | F: ACTGCCGCATCCTCTTCTC R: CTCTGCTTGTGATCCACATC | 53.8 | 29 | 399 |

F: Forward; R: Reverse.

recovery product was then connected to pMD-19T vector, and transformed to competent cells for sequencing.

Homology alignment and protein structure analysis for OLR1 in pig adipose tissue

First, the analysis of nucleotide homology alignment was carried out on NCBI web (<http://www.ncbi.nlm.nih.gov/>) using the Nucleotide BLAST function, then the OLR1 protein differences among related species were analyzed by Clustal X1.8 software, and thirdly, a phylogenetic tree was drawn by the Bootstrap Correction and Position Correction program from Mega2.1 software. Fourthly, the prediction of physical chemistry parameters of OLR1 protein was done with an online tool (<http://www.expasy.org/tools/>). Lastly, the prediction of OLR1 protein transmembrane structure and signal peptide was carried out using an online tool (<http://geome.cbs.dtu.dk/services/>) and the analysis of OLR1 protein structure was finished by online tools (<http://smart.embl-heidelberg.de/> and <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>).

Detection of OLR1 expression abundance in different tissues of pigs

According to the sequencing result of complete OLR1 cDNA, we designed OLR1 gene partial CDS primers and amplified OLR1 and β -actin genes from heart, liver, spleen, lung, kidney, muscle, visceral adipose and subcutaneous adipose of 10 MA LW pigs. Then the amplification products were detected by 1% agarose gel electrophoresis and images were analyzed by the SynGene Bio-image system and the absorbance (A) value of each band was recorded.

Detection of OLR1 expression level in adipose tissues at different MA and in different economic types of pigs

The expression levels of OLR1 and β -actin in subcutaneous adipose of 2, 5 and 10 MA LW and 5 MA GB pigs were detected using real time fluorescence quantitative PCR and the OD value of each band was recorded.

Detection of expression abundances of OLR1 and lipid metabolism related genes in adipose tissue of different economic types of pigs

The expression abundances of OLR1, PPAR γ 2, FAS, TGH, C/EBP α , SREBP-1c and β -actin were detected in subcutaneous adipose tissue of 5 MA LW and 5 MA GB pigs. After 1% agarose gel electrophoresis, the images were then analyzed by the SynGene Bio-image system and the OD value of each band was recorded. Then correlations between OLR1 and other genes were analyzed.

Data statistics and analysis

The experimental data were processed using SPSS 13.0

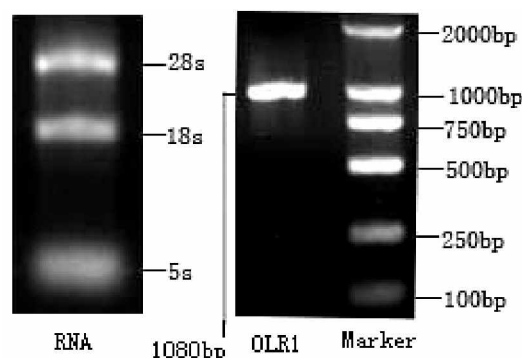


Figure 1. Electrophoresis results of total RNA and OLR1 PCR product from pig adipose tissue. The three bands of RNA in the image were clearly identified, which suggested the high quality of extracted RNA. The size of amplification product nearly matched with the expected fragment size.

software. Analysis of variance was carried out by the function: “Compare Means” → “One-Way ANOVA” → “LSD” method and the results were displayed by means \pm standard deviation (SD). Correlation analysis for the variances was carried out using the function: “Bivariate Correlation” → “Pearson Correlation” as well as “Graphs” → “Scatter/Dot...” → “Simple scatter”.

RESULTS

Cloning and sequencing of OLR1 gene in pig adipose tissue

We extracted total RNA from adipose tissue of GB pigs and amplified OLR1 cDNA complete CDS by RT-PCR, and the total RNA and amplification products were detected by agarose gel electrophoresis (displayed in Figure 1). The three bands of RNA in the image were clearly identified, which confirmed the high quality of extracted RNA and could be used. Further, the size of amplification product nearly matched with the expected fragment size, indicating that OLR1 gene complete CDS was successfully obtained from pig adipose tissue.

Next, we connected the amplification product to the vector for cloning, and then bidirectional sequencing was done. After removal of residues in N and C terminal non-translating regions by BLAST alignment to the sequencing analysis, we obtained pig adipose tissue OLR1 gene CDS which contained 825 bp and its accession No. in the GenBank is EU122440.

Analysis of OLR1 gene sequence in pig adipose tissue

We aligned the sequence of OLR1 gene CDS in pig adipose tissue with that of related species published in GenBank and found that the OLR1 gene in pig adipose tissue exhibited 99% homology to that in pig aortic endothelial tissue (NM_213805). The homology to cattle

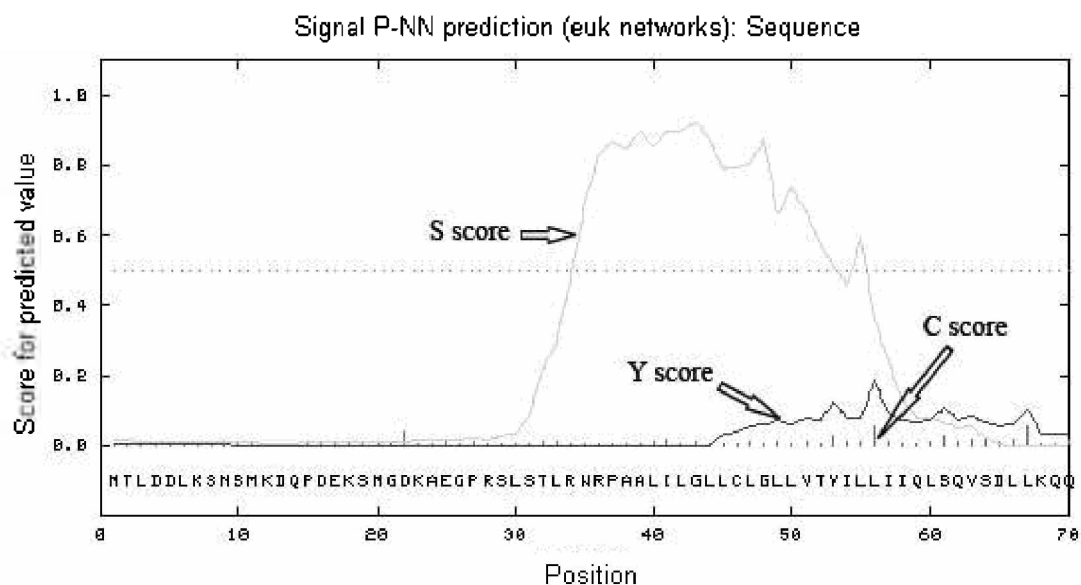


Figure 2. The signal peptide prediction of pig OLR1 protein. The high S score usually appears in the signal peptide area, and the low S score appears in the mature peptide area. The higher the S score is, the more likely it will be the signal peptide area. C score represents the evaluated score for cleavage site. Generally, the highest C score is predicted to be the cleavage site. Y score, which can accurately reflect the position of the cleavage site, is determined by both S score and C score.

(NM_174132), human (NM_002543), rat (NM_133306) and mouse (BC117712) was 84%, 79%, 51% and 27%, respectively. The *OLR1* in pig adipose tissue had three basyl transversions compared with that in pig aortic endothelial tissue (NM_213805), which were C308A, G402A and C401T, among which G402A was a silent mutation. Therefore, two amino acids were changed eventually: T103K and A137V.

The analysis for physical chemistry parameters of OLR1 protein revealed that OLR1 had a molecular weight of 31,197.8 Da and the theoretical PI was 6.60. Instability index was 47.20, which implied an instability characteristic for OLR1 protein, and the aliphatic index was 91.20, which demonstrated that globulin composition was high in OLR1 protein.

We predicted a signal peptide for pig adipose tissue OLR1 protein and the result is displayed in Figure 2. The prediction suggested that amino acid 30 to 64 probably contains a signal peptide area and the other areas were

considered to be mature protein. We also predicted transmembrane helices for OLR1 protein and the result demonstrated that the OLR1 protein has one transmembrane helix structure, which belongs to a type 2 membrane protein. Besides, amino acid 1 to 37 belonged to an intramembrane segment and amino acid 38 to 60 was likely to be a transmembrane helices area, while amino acid 61 to 274 belonged to an extramembrane section. According to the concrete score as well as the prediction result for the signal peptide, we can certify that amino acid 38 to 60 was determined to be a signal peptide area, exactly the same as the transmembrane helices section and the S score shown in Figure 2 had a high trend indicating that OLR1 protein was a secretory protein. The probable cleavage site should be in the middle of amino acid 55 to 56 (VIL-LI).

The analysis for domains and conserved domain of pig OLR1 protein is presented in Figure 3. There were three main functional domains in pig OLR1 protein: a transmembrane domain (38-60), a low complexity region

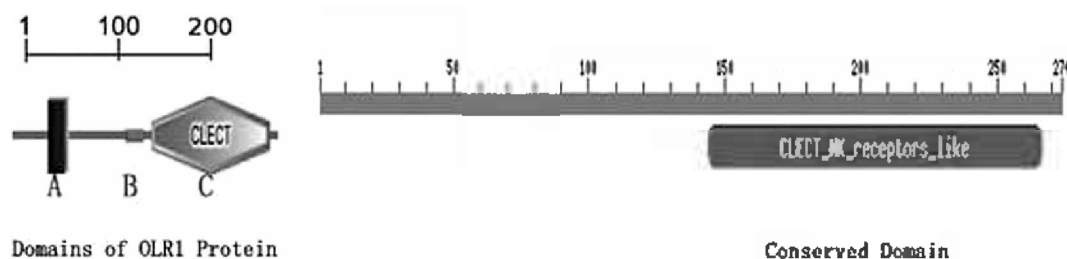


Figure 3. The domains and conserved domain prediction of pig OLR1 protein. A: C-type lectin domain, B: Low complexity region, C: C-type lectin or carbohydrate-recognition domain.

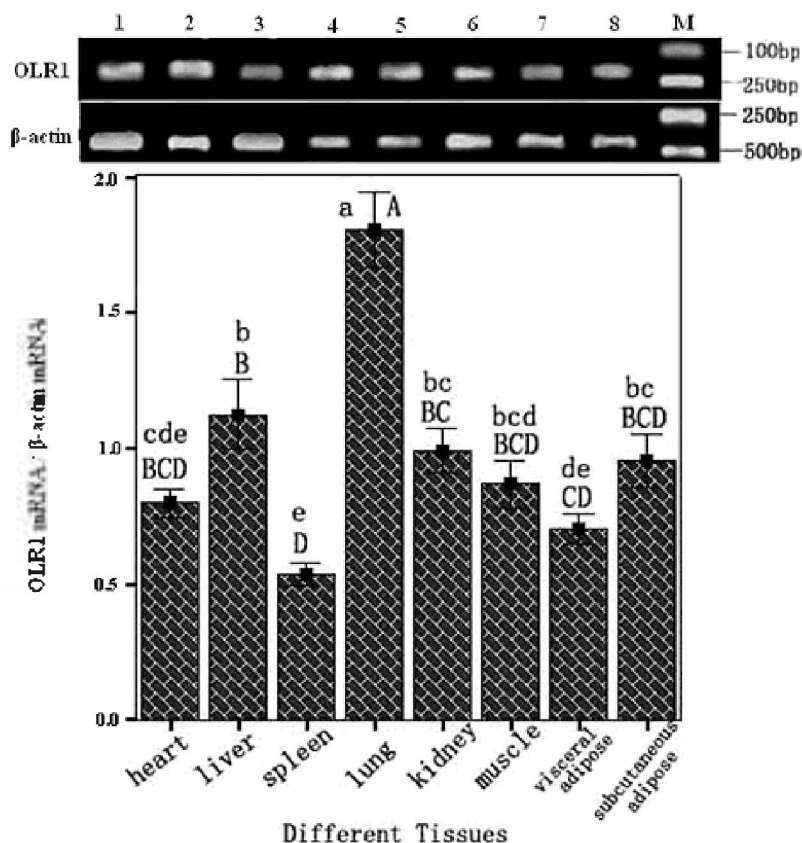


Figure 4. The amplification results of *OLR1* and β -actin and significance test of *OLR1* expression in different tissues of pigs. Values shown as means \pm SD of experiments from five animals, letters marked with a different superscript differ significantly (capital letters, $p < 0.01$; small letters $p < 0.05$). β -actin was used as an internal control. 1: heart, 2: liver, 3: spleen, 4: lung, 5: kidney, 6: muscle, 7: visceral adipose, 8: subcutaneous adipose, M: Marker.

(118-136), and a C-type lectin domain (or carbohydrate-recognition domain), but no glycosylation site was found. To combine the predicted results for domains and conserved domain, we concluded that amino acid 144 to 256 was the conserved domain comprising members of a C-type lectin family.

The expression abundance of *OLR1* gene mRNA in different pig tissues

Amplification of a 200 bp fragment of the *OLR1* gene in different pig tissues (Figure 4) indicated that the highest relative expression (compared to β -actin) was in the lung and the lowest was in the spleen and visceral adipose tissue, whereas expression in other tissues was at intermediate levels. Expression abundance in subcutaneous adipose tissue was significantly higher than that in visceral adipose tissue ($p < 0.01$). Because subcutaneous adipose has a stronger lipid deposition effect than visceral adipose (Mathieu et al., 2006), we can suppose that *OLR1* may play an important role in lipid deposition of subcutaneous adipose.

The expression characteristic of *OLR1* in adipose tissue

at different MA and in different economic types of pigs

Real time fluorescence quantitative PCR was employed to detect the expression abundance of *OLR1* and inner-reference β -actin in adipose tissues of 2, 5 and 10 MA LW and 5 MA GB pigs. With increasing age (MA), the expression of *OLR1* mRNA in pig adipose tissue increased significantly ($p < 0.01$) (Figure 5), which reflected that the expression abundance of *OLR1* had a close relationship with the amount of lipid deposition. At the same MA (5 MA for equality), the expression abundance of *OLR1* mRNA in adipose tissue of obese-type pigs (GB) was significantly higher ($p < 0.05$) than in lean-type pigs (LW) (Figure 5), which further illustrated that *OLR1* expression abundance was related to a higher accumulation of adipose tissue.

Analysis for expression correlation between *OLR1* and lipid metabolism related genes in pig adipose tissue

In order to further explore the role of *OLR1* in lipid deposition, we investigated the correlation between the relative expression of *OLR1* mRNA and the mRNAs of five lipid metabolism and adipogenesis related genes, *PPAR γ 2*, *FAS*, *SREBP-1c*, *C/EBP α* and *TGH*. Results revealed that, in pig adipose tissue, *OLR1* exhibited a significantly

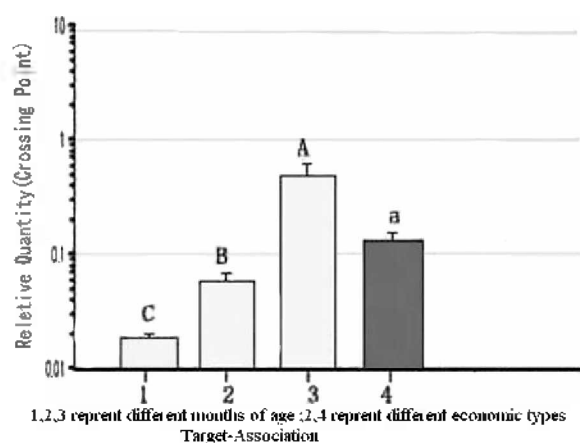


Figure 5. The *OLR1* expression rule in pig adipose tissues at different MA and in different economic types. NO.1-3 represent LW pig and NO.4 represents GB pig. 1: 2 MA. 2, 4: 5 MA. 3: 10 MA. Real-time quantitative PCR was used to detect the expression levels of *OLR1*. β -actin was used as an internal control. Values on Y-axis were obtained by the comparison of fluorescence intensity of *OLR1* and β -actin. Values shown as means \pm SD of experiments from five animals. Values marked with a different letter differ significantly (capital letters $p < 0.01$; small letters $p < 0.05$).

positive correlation with *PPAR γ 2* ($R = 0.928$, $p < 0.01$, Figure 6A) as well as *FAS* ($R = 0.863$, $p < 0.01$, Figure 6B) and *SREBP-1c* ($R = 0.746$, $p < 0.05$, Figure 6E). However, the correlations with *TGH* and *C/EBP α* were not statistically significant ($p > 0.05$, Figure 6C and D).

DISCUSSION

Oxidized low-density lipoprotein receptor 1 (*OLR1*) was first discovered in cattle aortic endothelial cells in 1997 (Sawamura et al., 1997). Since then, *OLR1* has been of interest because a close relationship between *OLR1* and atherosclerosis has been acknowledged (Chen and Du, 2007). Here we reported that *OLR1* was expressed in adipose tissue and was highly correlated with the expression of *PPAR γ 2*, *SREBP-1c* and *FAS*, suggesting a potential involvement with lipid deposition. The *OLR1* is a type II membrane protein that structurally belongs to the C-type lectin family with a short intracellular N-terminal hydrophilic and a long extracellular C-terminal hydrophilic domain separated by a hydrophobic domain (Chen et al., 2002). The lectin-like domain of *OLR1* protein is highly conserved among different species and is considered to be a ligand-recognition area which plays an essential role for ligand binding to protein (Chen et al., 2001). In our study, we also found this conserved domain, so the lectin-like domain is presumed to structurally influence the regulation of *OLR1* in lipid metabolism of different tissues as well as different species. The *OLR1* protein collected from pig

adipose tissue belongs to a secretory protein and amino acid 38-60 is a signal peptide.

We aligned the *OLR1* CDS of pig adipose tissue with those of related species and found that *OLR1* of pig adipose tissue has the highest homology with the cattle (84%), and the second highest is with human (79%). This outcome probably results from *OLR1* protein of the human possessing more functional domains and more complicated structures than in the pig and the cattle (Yamanaka et al., 1998). We also compared the *OLR1* CDS in pig adipose tissue with that in pig aortic endothelial tissue (NM_213805) and found that the *OLR1* in pig adipose tissue has three basal transversions and two amino acids changed eventually: T103K and A137V. Besides, these two amino acids just locate in the transmembrane helices area resulting in an alteration of structure, which may influence function. Whether this change is due to the specificity among tissues and actual difference in function need further investigation.

As no research on the expression characteristics of *OLR1* in pig tissues has been reported, we studied the expression abundance changes of *OLR1* in various pig tissues. The highest relative expression was in lung, which corresponded to observations on cattle tissues (Yamanaka et al., 1998). The expression levels of *OLR1* in liver, kidney, muscle and subcutaneous adipose did not show significant differences. We specially focused on the expression characteristic of *OLR1* mRNA in adipose tissue where *OLR1* had a significantly higher expression in subcutaneous than in visceral adipose tissue. Subcutaneous adipose tissue is the main depot in pigs (Laplante et al., 2006).

In order to verify the association of the expression of *OLR1* with lipid accumulation, we studied the expression of *OLR1* in subcutaneous adipose tissue from pigs at different months of age and of different economic types. Results showed that the expression of *OLR1* in the 5-month-old LW pig was significantly higher than at 2 months of age, and was significantly lower than in the 10-month-old LW pig ($p < 0.01$). Reiter et al. (2007) elucidated that anabolism-related genes display their higher expressions in obesity-type than in lean-type pigs, while in the adipose tissue of the lean-type pig the catabolism related genes display their advantages. Chui et al. (2005) reported that in the adipose tissue of an obese mouse model the expression of *OLR1* was significantly higher than in the normal controls. We also found that the expression of *OLR1* in obese pigs was higher ($p < 0.05$) at the same age (5-month-old) than in lean pigs. These observations indicated that *OLR1* may play an important role in lipid metabolism.

The *OLR1* has affinity for multiple ligands and exhibits complex biological functions. Experiments *in vitro* indicate that the *OLR1* protein can be induced to express by a variety of factors (Chen and Du, 2007). There is no

consensus for the OLR1 signal transduction pathway. Chen et al. (2007) indicated that OLR1 acts to influence atherosclerosis through the P38MAPK, ERK1/2 and PI3K pathway and many factors were involved in this process. However, the exact mechanism still needs to be approached. In mouse adipocytes, Chui et al. (2005) indicated that the *OLR1* promoter region contained a peroxisome proliferator-activated response element (PPRE) suggesting

transcriptional regulation by *PPAR γ* . In porcine subcutaneous adipose tissue, expression of *OLR1* was highly correlated with expression of *PPAR γ* . The *SREBP-1c* is another transcription factor closely associated with adipocyte differentiation, fatty acid metabolism and lipid deposition (Roder et al., 2007); in pig adipose tissue, *SREBP-1c* and *OLR1* were highly correlated. The expression level of *SREBP-1c* is 3.5 times greater in porcine

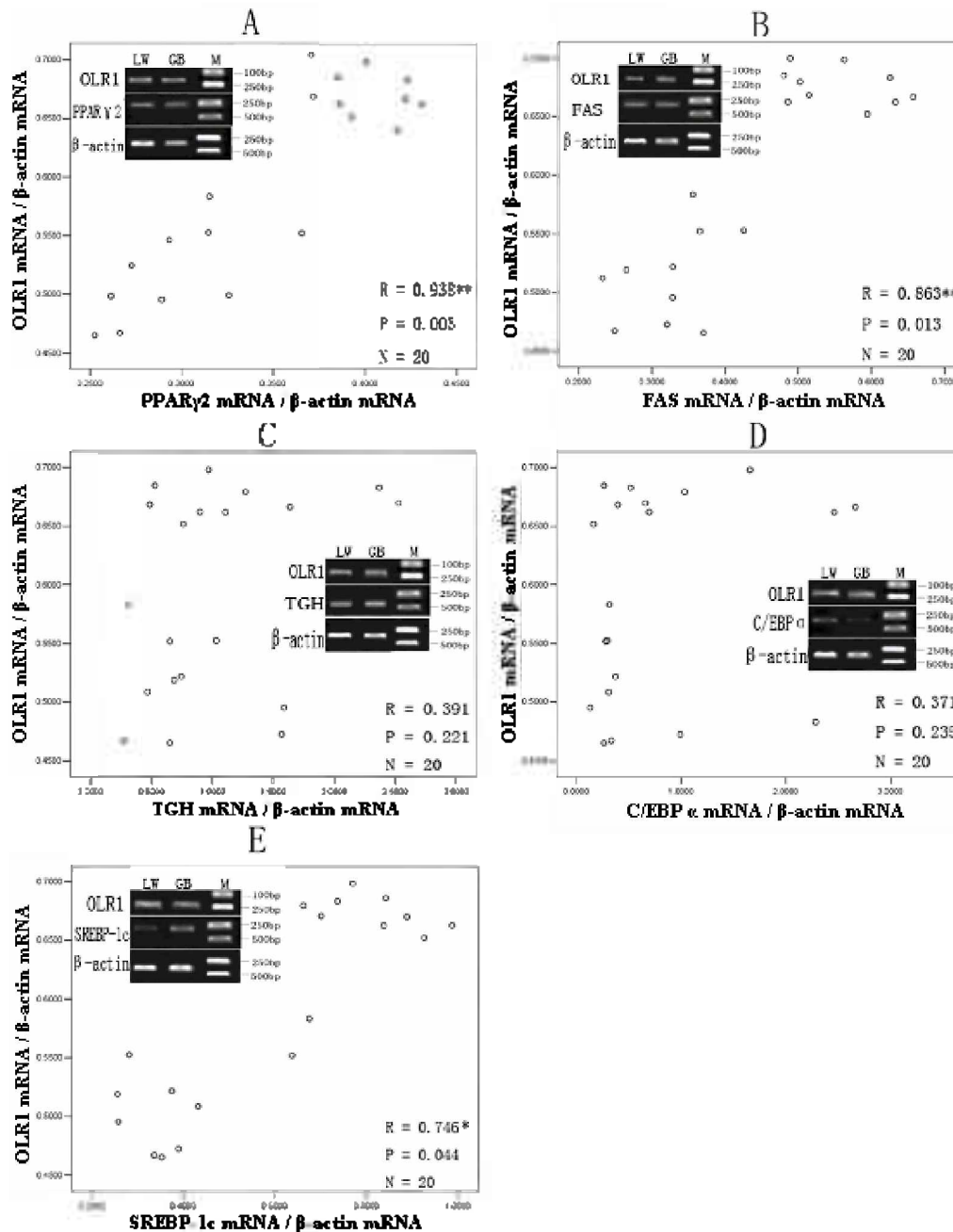


Figure 6. The amplification and correlation result of *OLR1* to other lipid metabolism related genes in different varieties of pig adipose tissues. "LW" represents LW pig; "GB" represents GB pig; "M" represents Marker; "R" represents Pearson correlation; "P" represents significant level; "N" represents sample number. Values are mean \pm SD of experiments from ten animals, each has two repetitions. Values in the figure represent the correlation of *OLR1* and other genes in pig adipose tissue. The "***" indicates $p < 0.01$; The "*" indicates $p < 0.05$.

adipose tissue (the primary site of fatty acid biosynthesis in this species) than in liver (Gondret et al., 2001). The expression of *OLRI* also was correlated with expression of *FAS*. A *SREBP-1c* binding site has been reported in the promoter region of *FAS* suggesting control of transcription by *SREBP-1c* (Schweizer et al., 2002).

In our experiment we did not observe a correlation between the expression of *OLRI* and that of *C/EBP α* in adipose tissue. In differentiating porcine adipocytes, the expression of *C/EBP α* is already elevated before visible lipid accumulation suggesting that *C/EBP α* is not a limiting transcription factor in this species (Ronald et al., 2000; Hausman, 2003). Although not definitively established, it is widely suggested that *TGH* plays an important role in adipose tissue catabolism. In adipocytes, Wei et al. (2007) demonstrated that down-regulation of the expression of *TGH* is accompanied by the accumulation of triglyceride and cholesterol esters. We found no correlation between expression of *OLRI* and *TGH*.

In summary, *OLRI* was increased with age and fat accumulation in porcine adipose tissue. This process was accompanied by an increased expression of *FAS*. The expression of *OLRI* in adipose tissue was positively correlated with expression of two key adipocyte transcription factors *PPAR γ 2* and *SREBP-1c*. However, expression of *OLRI* was not correlated with expression of *C/EBP α* , another important adipocyte transcription factor. The *OLRI* protein may be involved in anabolic lipid metabolism and lipid deposition, but at present, definitive conclusions cannot be drawn.

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