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Investigation of Genes Related to Porcine Fatty Deposition by Illumina Sequencing

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In this study, Illumina sequencing was used for transcriptome analysis of subcutaneous adipose and intramuscular adipose from exotic breeds (Landrace pigs) and domestic breeds (Taihu pig). For each library,1961 differentially expressed (DE) genes of the subcutaneous adipose between Landrace and Taihu pigs were identified. Fifty-four DE genes had functions described in the GO database and 15 DE genes were involved in the PPAR signaling pathway and fatty acid metabolism pathway described in the KEGG pathways. Comparison of intramuscular adipose between Landrace and Taihu pigs revealed 1219 DE genes. Fifty-five DE genes had functions described in the GO database and 12 DE genes were involved in the PPAR signaling pathway and fatty acid metabolism pathway. Digital analysis expression by tag profiling was a powerful approach to comprehensive transcriptome analysis to identify changes associated with fatty deposition, leading to a better understanding of the underlying mechanism of pathogenesis of fatty deposition.

Key words: Subcutaneous adipose, Intramuscular adipose, Differentially expressed gene; Pig.

Pork is the most commonly consumed meat worldwide[1]. Pig has become an important animal as one of the major sources of animal protein for humans[2]. Fat is an important indicator of meat quality, and the determining factor of the tenderness, flavor and juiciness of pork meat. Different varieties of pigs have different content and distribution of fat. For example, the intramuscular fat composition of Duroc (colored varieties) is much higher than white varieties pig, and the color, flavor and juiciness of Duroc are also much better than white varieties pig[3]. The polyunsaturated fatty acids composition in intramuscular fat of Crossbred pigs (Iberian * Duroc) is much higher than pure Iberian pigs, thus the intramuscular quality of Crossbred pigs is worse than pure Iberian pigs[4]. In pigs, high heritability has been estimated for backfat thickness*(BFT) and intramuscular fat (IMF) content[5]. IMF content and composition play an important role in both meat eating quality, particularly in the production of premium products, like high quality dry-cured ham, and human health. It has been shown that IMF content, although within limits, has a favorable effect on pork sensorial quality and that some specific fatty acids may have beneficial cardiovascular properties. Several studies reported on the benefits, for human health, of decreasing dietary saturated fatty acids (SFA) and the polyunsaturated (PUFA) n-6/n-3 fatty acid ratio while increasing monounsaturated (MUFA) fatty acids[6].In the past, lean percentage was thought to be due to breeding, and foreign

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lean type pigs were introduced, causing the decline of quality in pig meat. With changes to meat consumption, improving quality has become the focus of livestock genetics and breeding. Attempts at improving meat have been focused on breeding, resulting in the study on fat deposition-related genes, an area still in its infancy. Recently, scientists from China separated and cloned more than 40 new genes that closely related to pig meat quality and growth traits (FHL3, TNNI1, GYS1, ACOX1, FATP4, GBE1, HK2, etc.), and studied the relationship of these genes with carcass traits and meat quality. Despite such achievements in this field, there are still many problems. There is a lack of large-scale and systematic screening of key genes, and the function of most of the key genes is unclear, therefore, the findings cannot fully determine the genetic regulation of meat quantity and quality traits. Therefore, discovering the key genes that influence muscle growth and fat deposition, determining their function in fat deposition, and thus providing a rich source of genes for the transgenic technology of meat quality traits, is particularly important, urgent and significant. In addition, this work will not only help to protect the human diet health, but also have helping for the depth study of global disease – obesity, which caused by excessive deposition of fat.

So far, there have been many achievements in the scientific research on fat deposition, but the approaches used have several inherent limitations[7], such as expressed sequence tag (EST) sequencing, messenger ribonucleic acid (mRNA) differential display,subtractive hybridization and differential hybridization etc. The studies reported to date have made a great contribution towards identifying differentially expressed (DE) genes, but because they were unable to completely depict gene expression patterns, coupled with flux limitations, they have failed to meet the needs of large scale genetic screening and cloning. Recently developed RNA deep sequencing technologies, such as Solexa/ Illumina (San Diego, CA, United States) RNA-seq and digital gene expression (DGE), have dramatically changed the way immune-related genes in pig are identified because these technologies facilitate the investigation of the functional complexity of transcriptomes[8]. This method has an advantage over microarrays in that no probes are required, and it is especially more powerful in detecting differences in the low intensity range, compared to microarray platforms; hence, all transcripts can potentially be identified[9]. In this study, a new and potentially more comprehensive approach was applied: DGE tag profiling using Illumina sequencing to identify differentially expressed transcripts in adipose tissue of pigs from both exotic (Landrace Pigs) and domestic breeds (Taihu Pigs).

MATERIALS AND METHODS

Sample Preparation and RNA Extraction

Porcine adipose tissue samples were obtained from exotic breeds (Landrace Pigs) and domestic breeds (Taihu Pigs) all aged 150 days ,weighted 95±1kg. The pigs were slaughtered by electrical stunning. At the two pig breeds, fattening period ,we dissected and collected subcutaneous adipose and intramuscular adipose from the reciprocal ribs that between the third and fourth; samples were frozen in liquid nitrogen and stored at -80°C. The total RNAs were isolated from the tissues by Trizol (Tiangen Biotech Company, Beijing, China). The quality and quantity of RNA were determined using 1% agarose gel electrophoresis and Agilent bioanalyzer (Santa Clara, CA, United States). The results suggested that the purity of RNA extracted was suitable for the next step.

Sequence Tag Preparation

The Illumina sequencing process included sample preparation and sequencing. Beads of Oligo (dT) were used to enrich mRNA from the total RNA, and then were transferred into double-stranded copy deoxyribonucleic acid (cDNA) through reverse transcription. A 4 base-pair (bp) recognition enzyme, NlaIII, was used to digest the cDNA, and then Illumina adaptor 1 was ligated. Mmel was used to digest at 17 bp downstream of a CATG site; Illumina adaptor 2 was ligated at the 3' end. Primer GX1 and Primer GX2 were added for the polymerase chain reaction (PCR). Then, 95 bp fragments were regained through 6% Tris/Borate/EDTA (TBE) polyacrylamide gel electrophoresis (PAGE). The DNA was purified and followed by Illumina sequencing (Illumina Cluster Station and Illumina HiSeq™ 2000 system).

Gene Expression Annotation

Raw sequences have 3' adaptor fragments as well as a few low-quality sequences and several types of impurities. Raw sequences were transformed into Clean Tags after certain steps of data-processing by Geospiza software (Seattle, WA, United States). Virtual libraries containing all the possible CATG + 17 bp length sequences were searched for reference gene sequences (if there were no reference gene sequences for the species, sequences from closely related species were used for reference in Swissprot databases). All clean tags were mapped using Geospiza software to the reference sequences and only a 1 bp mismatch was considered. Clean tags mapped to reference sequences from multiple genes were filtered. Remaining clean tags were designated as unambiguous clean tags. The number of unambiguous clean tags for each gene was calculated and then normalized to TPM (number of transcripts per million clean tags).

Screening and Analysis of Differentially Expressed Genes

Referring to the significance of DGE profiles, we screened the DE genes. In our study, we used false discovery rate(FDR)≤0.001 and the absolute value of log 2 ratio ≥ 1 as the threshold to judge the significance of gene expression difference. Then, we determined the main biological functions of the DE genes by Gene Ontology (GO) functional enrichment analysis using the GSEABase software (http://www.r-project.org/). Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in differentially expressed genes compared with the whole genome background. At the end of the experiment, we performed a Pathway Enrichment Analysis for differentially expressed genes through the Gene Microarray Pathway Profiler(GenMAPP v2.1) (Gladstone Institute of Cardiovascular Disease,San Francisco,United States).

RESULTS

Sequencing Data Analysis

In this study, gene expression in subcutaneous adipose tissue and intramuscular adipose tissue in Taihu pigs and Landrace Pigs was assessed from tag libraries and analyzed by Illumina sequencing, giving 6,163,724 and 5,841,436 reads from replicate subcutaneous adipose tissue libraries (A and B; Taihu and Landrace, respectively) and 5,956,730 and 5,991,260 reads from replicate intramuscular adipose tissue libraries (C and D; Taihu and Landrace, respectively) (Fig.1). Tags containing N, only adaptor tags, copy number <2 and low-quality tags were filtered out for clean tags. A total of more than 5 million tags were generated from each group and, moreover, approximately 60–67% of the total reads mapped to the gene and 6% of the tags were identified in the genome. Subsequently, consensus sequences were merged for DGE analysis.

Differential Expression of Gene Number

BLASTX software analysis of non-redundant tags revealed that the reliable coding sequences had high potential for translation into functional proteins and most of them translated to proteins with more than 100 amino acids. Comparison with the Nr and Swissprot databases revealed that in subcutaneous adipose tissue, the number of differentially expressed genes between Taihu and Landrace Pigs was 1961, including 1279 up-regulated genes and 682 down-regulated genes; the number of differentially expressed genes in the intramuscular adipose tissue of Taihu and Landrace pigs is 1219, including 563 up-regulated genes and 656 down-regulated genes (Fig. 2).

Differential Gene Expression: Significant Enrichment Analysis (Pathway)

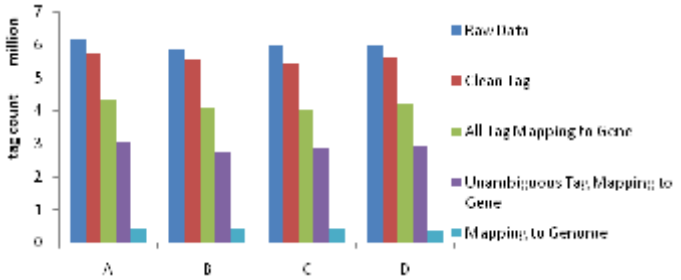
To gain deeper insight into the molecular biology of genes in adipose tissues, we performed a GO function analysis in DE genes, including 1961 genes in subcutaneous and 1219 genes in intramuscular adipose tissue. GO-annotated consensus sequences identify the biological process, cellular component, and molecular function. In total, 1035 DE genes of subcutaneous adipose tissue and 671 DE genes of intramuscular adipose tissue had functions described in the GO database. In subcutaneous adipose (Fig. 3), the major categories identified in GO analyses were: other biological processes (156 DE genes), other metabolic processes (110 DE genes), developmental processes (88 DE genes), RNA metabolism (84 DE genes), protein metabolism (82 DE genes), cell organization and biogenesis (71 DE genes), transport (62 DE genes), stress response (52 DE genes) and signal transduction (65 DE

Table 1. Differentially Expressed Genes of Subcutaneous Adipose Tissue in the KEGG Pathway Database

Name of pathway	Count	Up-count	Down-count
PPAR signaling	10	7	3
Renal cell carcinoma	10	6	4
Arginine and proline metabolism	8	6	2
Complement and coagulation cascades	8	7	1
Glutathione metabolism	6	5	1
Fatty acid metabolism	5	4	1
Histidine metabolism	4	3	1
Butanoate metabolism	4	3	1
mTOR signaling	5	2	3
Metabolic pathways	48	35	14
beta-Alanine metabolism	3	3	0
MAPK signaling pathway	15	11	4
Toll-like receptor signaling	7	5	2
Notch signaling	4	2	2
Hematopoietic cell lineage	6	5	1
Adipocytokine signaling	5	1	4
Glycine, serine and threonine metabolism	3	3	0
Apoptosis	6	3	3
Terpenoid backbone biosynthesis	2	1	1
Cell cycle	7	3	4
Lysosome	7	6	1
Propanoate metabolism	3	2	1
Adherens junction	5	4	1
Arachidonic acid metabolism	4	3	1
Glycosaminoglycan degradation	2	2	0
Pyruvate metabolism	3	1	2
T-cell receptor signaling	6	4	2
TGF-beta signaling	5	2	3
ErbB signaling	5	3	2
N-Glycan biosynthesis	3	2	1
ABC transporters	3	2	1
Insulin signaling	7	4	4
Tyrosine metabolism	3	3	0
Selenoamino acid metabolism	2	2	0
Focal adhesion	9	6	3
B cell receptor signaling	4	3	1
Citrate cycle (TCA cycle)	2	0	2
Cytokine-cytokine receptor interaction	11	7	4
DNA replication	2	1	1
Tryptophan metabolism	2	2	0
Wnt signaling	6	5	1
Purine metabolism	6	5	2
Amino sugar and nucleotide sugar metabolism	2	2	0
Neurotrophin signaling	5	3	2
Jak-STAT signaling	6	4	2
Valine, leucine and isoleucine degradation	2	2	0
Oxidative phosphorylation	5	4	2
Glycerolipid metabolism	2	2	0
VEGF signaling	3	1	2
Lysine degradation	2	2	0

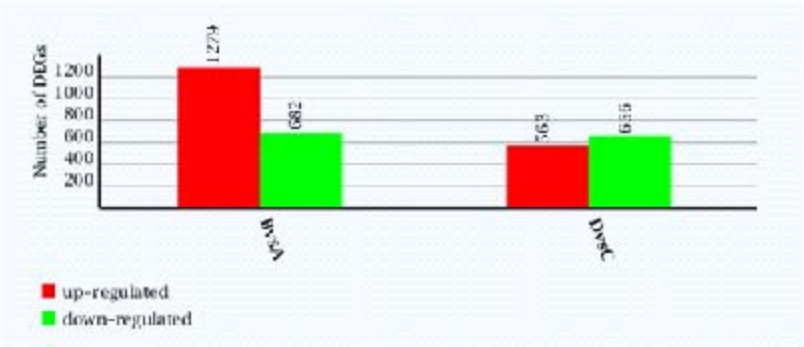
Regulation of actin cytoskeleton	7	5	2
Endocytosis	6	4	2
Glycolysis / Gluconeogenesis	2	1	1
Axon guidance	4	3	1
Chemokine signaling	6	3	3
Tight junction	4	2	2
Ubiquitin mediated proteolysis	4	2	2
Drug metabolism - cytochrome P450	2	2	0
Calcium signaling	5	4	1
FCεRI signaling	2	1	1
Leukocyte transendothelial migration	3	3	0
ECM-receptor interaction	2	1	1
Antigen processing and presentation	2	1	1
GnRH signaling	2	2	0
Cell adhesion molecules (CAMs)	2	2	0
Natural killer cell mediated cytotoxicity	2	2	0
Neuroactive ligand-receptor interaction	3	1	2

PPAR=peroxisome proliferator-activated receptors; mTOR=mammalian target of rapamycin; MAPK=mitogen-activated protein kinase; TGF=transforming growth factor; ErbB=epidermal growth factor receptor; ABC=ATP-binding cassette; TCA=tricarboxylic acid cycle; JAK=janus kinase; STAT=signal transducer and activator of transcription; VEGF=vascular endothelial growth factor; FCεRI=high-affinity immunoglobulin E receptor; ECM=extracellular matrix; GnRH=gonadotropin releasing hormone



A is the subcutaneous adipose tissue of the Taihu pigs. B is the subcutaneous adipose tissue of the Landrace pigs. C is the intramuscular adipose tissue of the Taihu pigs. D is the intramuscular adipose tissue of the Landrace pigs

Fig. 1. Summary of the Mapping Results From the Replicate Libraries Prepared From Subcutaneous Adipose Tissue and Intramuscular Adipose Tissue Between Taihu Pigs and Landrace Pigs After Tag Sequencing.



DEGs=differentially expressed genes; A is the subcutaneous adipose tissue of the Taihu pigs. B is the subcutaneous adipose tissue of the Landrace pigs. C is the intramuscular adipose tissue of the Taihu pigs. D is the intramuscular adipose tissue of the Landrace pigs. A compare result AvsB, where A is the control, B is the treatment. C compare result CvsD, where C is the control, D is the treatment.

Fig. 2. Differentially Expressed Genes

Table 2. Differentially Expressed Genes of Intramuscular Adipose Tissue in the KEGG Pathway Database

Name of pathway	Count	Up-count	Down-count
Metabolic pathways	33	12	22
PPAR signaling	8	3	5
MAPK signaling	8	4	4
Cytokine–cytokine receptor interaction	8	5	3
Hypertrophic cardiomyopathy (HCM)	7	0	7
Toll-like receptor signaling	6	3	3
Insulin signaling	6	2	4
Adipocytokine signaling	6	2	4
Oxidative phosphorylation	5	3	3
Chemokine signaling	5	2	3
Apoptosis	5	2	3
Tight junction	5	0	5
Hematopoietic cell lineage	5	4	1
Neurotrophin signaling	5	3	2
Fatty acid metabolism	4	1	3
Glutathione metabolism	4	1	3
Pyruvate metabolism	4	0	4
Calcium signaling	4	1	3
Lysosome	4	4	0
TGF-beta signaling	4	3	1
Jak-STAT signaling	4	3	1
Glycolysis / Gluconeogenesis	3	0	3
Purine metabolism	3	1	2
Arginine and proline metabolism	3	2	1
Glycerophospholipid metabolism	3	1	2
Drug metabolism - cytochrome P450	3	1	2
Neuroactive ligand-receptor interaction	3	2	1
Ubiquitin mediated proteolysis	3	2	1
Citrate cycle (TCA cycle)	2	0	2
Pentose phosphate	2	0	2
Valine, leucine and isoleucine degradation	2	0	2
Histidine metabolism	2	0	2
Starch and sucrose metabolism	2	1	1
Amino sugar and nucleotide sugar metabolism	2	2	0
Glycerolipid metabolism	2	0	2
Retinol metabolism	2	0	2
Porphyrin and chlorophyll metabolism	2	2	0
Metabolism of xenobiotics by cytochrome P450	2	1	1
Biosynthesis of unsaturated fatty acids	2	1	1
Biosynthesis of alkaloids derived from ornithine, Lysine and nicotinic acid	2	0	2
ABC transporters	2	1	1
ErbB signaling	2	1	1
p53 signaling	2	0	2
Endocytosis	2	1	1
Wnt signaling	2	1	1
Hedgehog signaling	2	2	0
Focal adhesion	2	0	2
Regulation of actin cytoskeleton	2	1	1

PPAR=peroxisome proliferator-activated receptors; MAPK=mitogen-activated protein kinase; TGF=transforming growth factor; JAK=janus kinase; STAT=signal transducer and activator of transcription; TCA=tricarboxylic acid cycle; ABC=ATP-binding cassette; ErbB=epidermal growth factor receptor

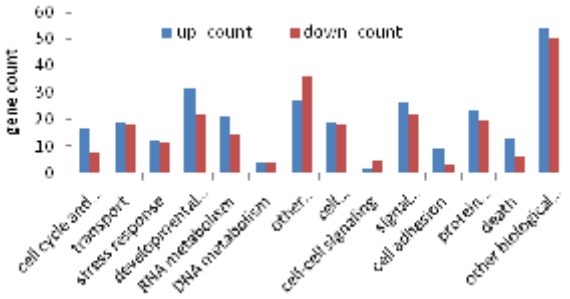


Fig. 3. Differentially Expressed Genes Involved in the GO Biological Process Analysis in Subcutaneous dipose

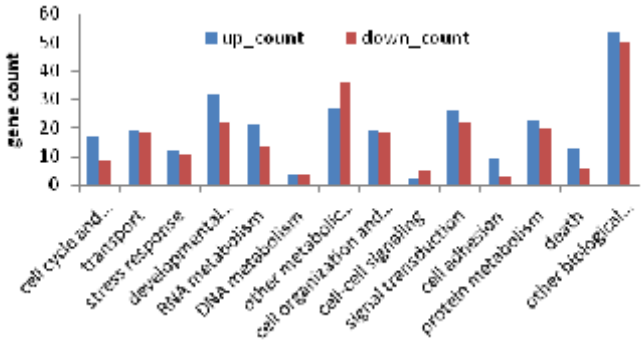
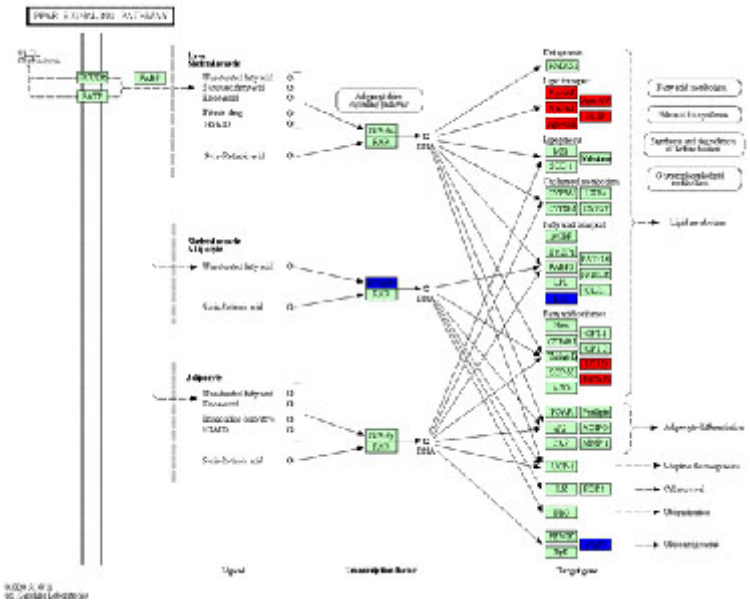


Fig. 4. Differentially Expressed Genes Involved in the GO Biological Process Analysis in Intramuscular Adipose

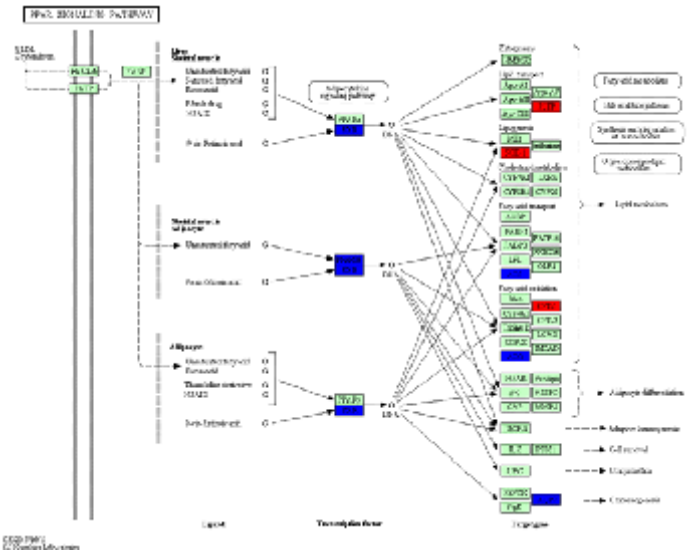


Up-regulated genes are marked in red, down-regulated genes marked in blue. Light green marks genes of no special significance. PPAR=peroxisome proliferator-activated receptors

Fig. 5. The PPAR Signaling Pathway in Subcutaneous Adipose Tissue

genes). In intramuscular adipose tissue (Fig. 4), there were 14 genes clusters that had gene enrichment, these included: other biological processes (103 DE genes), other metabolic processes (63 DE genes), signal transduction (48 DE genes), protein metabolism (43 DE genes), cell organization and biogenesis (37 DE genes).

In subcutaneous adipose tissue, 353 of the DE genes were found to be involved in 68 different pathways included in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Table 1). The major pathways detected in this study include the metabolic pathways, mitogen-activated protein kinase (MAPK) signaling, cytokine–cytokine receptor interaction, peroxisome proliferator-activated receptors (PPAR) signaling, renal cell carcinoma, arginine and proline metabolism, complement and coagulation cascades, cell cycle and focal adhesion. There were 202 DE genes, which were included in 49 different KEGG pathways, in intramuscular adipose tissue (Table 2). The major pathways also included the metabolic pathways, PPAR signaling, MAPK signaling and fatty acid metabolism. In previous studies, it was demonstrated that the PPAR gene was closely involved in fatty deposition in the pig[10]. In our study, we found seven up-regulated DE genes and three down-regulated DE genes in the PPAR signaling pathway in subcutaneous adipose tissue (Fig. 5), they were PLTP, ACADL, ACS, APOA2, APOA5, APOA1, APOC3, PPARD, PCK1 and AQP7. Three up-regulated DE genes (PLTP, SCD1 and CPT1A) and five down-regulated DE genes (AQP7, PPARD, RXRG, ACOX1, ACSL1) in the PPAR signaling pathway were found in intramuscular adipose tissue (Fig. 6).



Up-regulated genes are marked in red, down-regulated genes marked in blue. Light green marks genes of no special significance. PPAR=peroxisome proliferator-activated receptors.

Fig. 6. The PPAR Signaling Pathway in Intramuscular Adipose Tissue

DISCUSSION

Reduction of fat deposition has been a major goal in the continuing improvement of pork production for the last five or six decades[11]. Since the 1960s, the intramuscular fat content has been reduced from 2–4% to less than 1%[12]. As a result of demand from consumers, high heritability (h^2=0.5) and economic value, the genetic improvement of intramuscular fat is a current and continuing issue[13]. Moreover, excessive fat deposition will not only affect carcass quality, but greatly influence the economic interests of pig breeders. Because in the process of breeding pigs, excessive fat deposition would endanger the health of animals, such as cause cardiovascular diseases, disorder the digestive system and endocrine system, and mechanical damage caused

by obesity.More importantly, excessive ingestion of fat can cause obesity, diabetes, atherosclerosis and coronary heart disease, which is obviously not conducive to the healthy diet for humans. As a global disease, the incidence of obesity increases every year, this disease is due to excessive accumulation of body fat. Therefore, a clear molecular mechanisms of fat deposition is also significant for the research of obesity. Therefore, it is necessary to perform in-depth research on the fat metabolism and regulation, *in vivo*.

In our study, we recovered total RNA from the adipose tissue, and used the Illumina RNA-seq to identify the genes that may be related to pig fatty deposition. The transcriptome is the complete repertoire of expressed RNA transcripts in a cell. It is essential in distinguishing the functional complexity of the genome, and obtaining a better understanding of cellular activities in organisms, including growth, development, disease and immune defense. In addition, the newly developed Illumina RNA-seq and DGE high-throughput deep sequencing approaches have dramatically changed how functional complexity of the transcriptome can be studied. These measures surmount a lot of the inherent limitations of traditional systems, making the detection of alternative splicing events and low-abundance transcripts possible. Recently, they have been applied to several species, such as Zebrafish, Drosophila, humans, yeast, Arabidopsis, Chlamydomonas and Caenorhabditis[14-24].

We collected more than one thousand DE genes both in the subcutaneous adipose tissue and the intramuscular adipose tissue between Taihu pig and Landrace pigs, including 1035 DE genes of subcutaneous adipose tissue and 671 DE genes of intramuscular adipose tissue that had functions described in the GO database. At the end of the study, in subcutaneous adipose tissue, KEGG analysis showed that more than 18% of DE genes were enrichment factors involved in approximately 68 known metabolic or signaling pathways. In the intramuscular adipose tissue, 17% of DE genes were involved in 49 different KEGG pathways. After analyzing our data, we found the results showed some similarities with previous studies. Many researchers had previously found factors influencing the development of adipose tissue in the growing pig, including IGF2, UCP3, PPAR^3, ADIPOQ, POU1F1, SREBF1, GHRL, NUDT6, LDLR and RYR1[25-32]. However, our results found differences between species in the subcutaneous adipose tissue:for example,the LDLR gene was differentially expressed by multiples of 1.2309; the PPARD gene was differentially expressed by 1.2879of; and the NUDT6 gene was differentially expressed by 1.1036.In the intramuscular adipose tissue,the PPARγ gene was differentially expressed by 1.3728; the IGF2 gene was differentially expressed by 1.8143; and the RYR1 gene was differentially expressed by 2.0788.Our results not only validate the previous findings,but also highlight the differences between different domestic and international species more clearly, providing some basis for the improvement of domestic species.

Furthermore,we identified some genes that were differentially expressed between the two pig breeds and were involved in pathways that are associated with fat metabolism; these genes may be the key to fat deposition.For instance,in subcutaneous fat tissue,the gene coding for medium-chain specific acyl-CoA dehydrogenase (MCAD) was found to be differentially expressed by 1.051.The molecular function of MCAD is acryloyl-CoA reductase activity,located at the cell mitochondrion; this biological process is independently involved in fatty acid oxidation,lipid metabolism and mitochondrial fatty acid beta-oxidation.In intramuscular fat tissue, the retinoic acid receptor RXR-gamma (RXR) gene was differentially expressed by 2.8875. This protein is located in the cell nucleus and functions as a receptor;likewise,it participates in the PPAR signaling pathway.According to previous studies,RX compounds have important biological activity, especially for animal embryonic development and organ formation[33].There were also some genes that were differentially expressed by more than multiples of 10,for example,the MYOTI gene was differentially expressed by 10.1459,and the HIGD2A gene was differentially expressed by 10.8556 in subcutaneous adipose tissue.In intramuscular adipose tissue,the PCK1 gene was differentially expressed by 13.8336.The function of these genes should, therefore, be studied.

It was determined that the differential expression of these genes leads to thicker fat on the Taihu compared with the Landrace pigs. Following theses findings,we will proceed to test

these differentially expressed genes to verify their function.Similarly,the tags that have not matched to the gene also have great value. As the pig genome sequence map is not completely drawn,some new genes could be discovered through these unknown tags.

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