Identification of Key Pathways and Genes in the Subcutaneous Fat Tissue from Different Sexes of Pigs using WGCNA Method

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Abstract

Background: Obesity is a complex disease that will endanger human life and health, and obesity is closely related to subcutaneous fat deposition. Some studies have shown the significant association between subcutaneous fat deposition and sex. However, the molecular mechanisms for this association are still unclear. The objective of this study is to identify key pathways and genes related to the subcutaneous adipose tissue between different sexes of pigs.

Results: A total of 16 coexpression modules were detected, of which two sex-related modules were found. Functional enrichment analyses showed that the genes in the purple module were mainly enriched in some pathways related to lipid metabolism such as Regulation of lipolysis in adipocytes. The genes in the magenta module mainly involved in some pathways related to immunity such as Defense response to virus and Immune response. Furthermore, 7 extracellular matrix (ECM)-related genes and 7 lipid metabolism-related genes were identified in the purple module. In the magenta module, 17 genes and 2 transcription factors participating in Type I interferon response were identified which were associated with immunity.

Conclusions: The present study identified key pathways and genes in subcutaneous adipose tissue from different sexes of pigs, which provided some insights into the molecular mechanism involving in fat formation and immunoregulation between pigs with different sexes. These findings may be helpful for breeding in pig industry and obesity treatment in medicine.

Background

Obesity is a complex disease that will endanger human life and health. Pigs are important domestic animals and important biomedical models for human diseases, such as obesity, type II diabetes, and cardiovascular diseases, because their body size and physiological/anatomical features are similar to those of humans [1]. As far as we know, adipose tissue is a central metabolic tissue of complex and highly metabolically active which participates in regulating systemic energy homeostasis [2]. The subcutaneous adipose tissue of pigs is very important for the study of fat deposition in pigs and metabolic diseases for human such as obesity. At present, most of the studies mainly focused on the comparative study of differentially expressed genes (DEGs) in subcutaneous adipose tissue between different pig breeds, and identified some important pathways and genes related to fat deposition. For example, Huang et al. analyzed the gene expression profiles of subcutaneous adipose tissues in Laiwu and Large White pigs and found that DEGs were significantly enriched in PPAR signaling pathway and two biological processes, including fatty acid metabolism and fat cell differentiation [3]. Albuquerque et al. identified some important genes involved in *de novo* fatty acid biosynthesis, elongation and desaturation by analyzing the gene expression profiles of dorsal subcutaneous fat tissues in Iberian and Alentejano pig breeds, such as *ACLY*, *FASN*, *ME1*, *ELOVL6*, and *SCD* [4]. Lim et al. identified 136 DEGs in subcutaneous adipose tissue between the Korean native pig and Yorkshire using a cDNA microarray, and found that some DEGs were involved in fat metabolism processes such as adipogenesis, adipocyte differentiation and lipolysis [5]. Madeira et al. found that the mechanisms regulating fat deposition in pigs were genotype and tissue specific, and were associated with the expression regulation of the key lipogenic genes including *ACACA*, *CEBPA*, *FASN* and *SCD* [6].

In recent years, some studies showed that fat deposition and characteristics were influenced by the sex of pigs [7, 8]. Zhou et al. found that the fat cells of female pigs were larger in volume and the mRNA abundance of related genes was higher by comparing with Rongchang and Landrace male pigs in six different adipose tissues [9]. Mentzel et al. found that many differentially expressed miRNAs were significantly associated with gender by analyzing the expression profile of miRNAs in subcutaneous adipose tissues from male and female crossbred F2 of Göttingen minipigs, Duroc and Yorkshire pigs, which demonstrated that the fat deposition were potentially regulated by miRNAs between genders [10]. Despite some progress, the molecular mechanisms of fat formation involved in gender were still unclear. Especially, the gene coexpression relationship with the sex remained unknown.

Weighted Gene Coexpression Network Analysis (WGCNA) is a systematic biology method to describe the correlation patterns among genes across samples [11]. Compared with other methods, WGCNA focuses on the relationship between coexpression modules and phenotypes [12], and using WGCNA can find the gene coexpression modules with higher reliability and biological significance and identify “driver” genes in the modules [13]. Presently, WGCNA has become the most important way to study the coexpression relationships between genes and been successfully applied in various research fields, such as complex diseases including bladder cancer [14], lung squamous cell carcinoma [15], Hepatocellular carcinoma [16], uveal melanoma [17], hyperlipidaemia [18], obesity [19], and economic traits including meat quality [20], hypoxic adaptation [21] and skin color [22], and so on. Lim et al. identified functional modules and hub genes related to marbling trait in Hanwoo (Korean) cattle using WGCNA method, and these hub genes mainly involved in biological processes correlated with fat or muscle formation [23]. Xing et al. found that four coexpression modules were significantly correlated with backfat thickness in Songliao black and Landrace with high and low backfat using WGCNA method [24]. Besides, Protein and protein interaction (PPI) networks are also viable tools to construct a gene coexpression network and to understand cell functions and disease machinery [25]. However, interpreting a PPI network and identifying core proteins and genes are a particularly challenging task because of the network complexity. For this reason, some algorithms have been developed, including Molecular complex detection, cytoHubba and centiScape, and so on. Zhao et al. identified *ADIPOQ*, *PPARG*, *LIPE*, *CIDEA*, *PLIN1*, *CIDEA* and *FABP4* as potentially candidate genes affecting intramuscular fat (IMF) content in 28 purebred Duroc pigs by integrating the results from WGCNA and PPI methods [26].

In the present study, we applied the WGCNA and PPI network analysis method, using RNA-Seq data of subcutaneous fat tissue of male and female pigs (crossbred F2 of Duroc and Göttingen miniature pig) from Gene Expression Omnibus (GEO) database, with the aim to identify the significant modules closely related to sex of pigs, and further identify key pathways and genes. These findings may contribute to further understand the function of adipose tissue of pigs and the mechanisms regulating fat deposition in subcutaneous fat tissue from pigs with different sexes, and provide some insights into the obesity treatment in medicine. Moreover, the identified key genes may serve as the potential biomarkers in pig breeding and potential targets in obesity treatment.

Results

WGCNA and the significant module identification

The expression matrix containing 5000 genes were used to reconstruct the gene coexpression network using WGCNA method. Pearson correlation matrix among genes were converted into a strengthened adjacency matrix by power $\beta=5$ based on scale-free topology criterion with $R^2=0.9$ (Fig. 1a). Topological overlap measure (TOM) of each gene pair was calculated, and 16 gene coexpression modules were identified by average linkage hierarchical clustering according to TOM-based dissimilarity (1-TOM) (Fig. 1b). There were large differences in the number of genes between the modules, and the smallest midnightblue module contained 59 transcripts, while the largest turquoise module contained 914 transcripts. The purple module contained 227 transcripts, and the magenta module contained 254 transcripts (Table S1).

The eigengene adjacency heatmap depicting the interaction of the identified modules was showed in Fig. 1c, while the network heatmap plot of all the genes was showed in Fig. 1d. As shown in Fig. 1e, the correlation analysis between Module eigengene (ME) and sex showed that the purple module was the most significantly representative module ($\text{cor}=-0.73$ and $P=5e-07$), and the magenta module was the third significantly representative module ($\text{cor}=0.5$ and $P=0.002$). Gene significance (GS) analysis across modules ($P=2.2e-259$) showed that the purple module and magenta module had the highest and second highest GS, respectively (Fig. 1f). The module membership (MM) in the purple module possessed the most significant correlation across all modules ($\text{cor}=0.71$ and $P<4e-36$, Fig. 2a), and the MM in the magenta module possessed the second significant correlation across all modules ($\text{cor}=0.63$ and $P<1.7e-29$, Fig. 2b). Thus, the purple module and magenta module were selected for further analyses. The expression heatmap of all genes in the purple and magenta module were respectively showed in Fig. 2c and 2d.

GO and KEGG analysis and key genes identification

GO and KEGG enrichment analysis were performed on all genes in the purple module and magenta module using the Database for Annotation, Visualization and Integrated Discovery (DAVID) database. In the purple module, GO enrichment results showed that 9 biological processes (BPs), 7 cellular components (CCs) and 4 molecular functions (MFs) were significantly enriched ($P<0.05$). KEGG enrichment analysis showed that 6 KEGG pathways were significantly enriched ($P<0.05$). Top 1 BP, CC and MF terms and Top 4 KEGG pathways were showed in Table 1. KEGG pathway analysis showed that the genes in the purple module were significant enriched in Regulation of lipolysis in adipocytes, Aldosterone synthesis and secretion, Ras signaling pathway and MAPK signaling pathway, and so on. GO analysis showed that the most significant enriched CC term was Extracellular space. The most significant enriched BP term was Muscle organ morphogenesis. The most representative MF term was Heparin binding. In addition, the functional analysis for the magenta module was performed, GO and KEGG enrichment results showed that 23 BPs, 3 CCs, 11 MFs and 12 KEGG pathways were significantly enriched ($P<0.05$). Top 2 BPs, Top 1 CC and MF terms and top 4 KEGG pathways were showed in Table 2. In KEGG analysis, genes in the magenta module mainly enriched in pathways related to immunity disease, including Influenza A, Hepatitis C, Measles and Herpes simplex infection, and so on. The most significant enriched CC term was External side of plasma membrane, and the significant enriched BP terms were related to immunity process, including Defense response to virus and Immune response. The most representative MF term was Double-stranded RNA binding.

In the purple module, the selection criteria of key genes were that genes participated in two terms, and at least involved in one KEGG pathway. Besides, all the genes involved in the pathway of regulation of lipolysis in adipocytes were selected as key genes. So, 10 key genes, *LIPE*, *NPR1*, *AQP7*, *NPY1R*, *PLA2G16*, *LOC100522721*, *FGF10*, *FGFR2*, *CACNA1G* and *IGF1* were identified and showed in Fig. 3a. In the magenta module, the selection criterion of key genes was that genes involved in at least three terms. 12 genes, *FAS*, *TNFSF10*, *DDX58*, *MX1*, *IFIT1*, *ADAR*, *OAS2*, *OAS1*, *CXCL9*, *CXCL10*, *STAT1* and *IRF9* were selected as key genes (Fig. 3b).

PPI network construction and hub genes identification

All genes in the purple and magenta modules were used to perform PPI network analysis. At combined score > 0.4 , a PPI network including 116 nodes and 169 edges was constructed for the purple module (called the first PPI network), and was showed in Fig. 4a. For the magenta module, a PPI network containing 153 nodes and 814 edges was established and showed in Fig. 4b (called the second PPI network). Top genes with higher scores were identified by centrality analyses in the two modules using the centiScape. The result of centrality analysis for genes with top 10 centrality scores in the first PPI network and top 20 centrality scores in the second PPI network were respectively showed in Table 3 and Table 4. In the purple module, top 10 genes were *COL1A2*, *LOC100738989*, *MMP2*, *COL1A1*, *SPARC*, *POSTN*, *SERPINH1*, *IGF1*, *FMOD* and *FKBP10*, separately. In the magenta module, top 20 genes were *USP18*, *MX1*, *STAT1*, *ISG15*, *IRG6*, *MX2*, *IFIT3*, *IFIT2*, *IFIT1*, *DDX58*, *OAS1*, *PARP9*, *DDX60*, *IFI44L*, *LFI35*, *IFI44*, *PARP14*, *IRF9*, *XAF1* and *OAS2*, separately.

To further excavate the hub gene in the two modules, the cytoHubba was used to screen out hub genes in the whole PPI network. According the Maximal Clique Centrality (MCC) score, top 10 genes were selected as hub genes in the first PPI network. A sub-network including 10 nodes and 36 edges in the first PPI network was identified and showed in Fig. 4c. These hub genes were *COL1A2*, *MMP2*, *COL1A1*, *SPARC*, *POSTN*, *SERPINH1*, *FMOD*, *ENSSSCG00000017581*, *ENSSSCG00000029074* and *LOC100738989*. According to the MCC score, top 20 genes were selected as hub genes in the second PPI network. A sub-network containing 20 nodes and 187 edges in the second PPI network was identified and showed in Fig. 4d. These hub genes were *USP18*, *MX1*, *STAT1*, *ISG15*, *IRG6*, *ENSSSCG00000027918*, *MX2*, *IFIT3*, *IFIT2*, *IFIT1*, *DDX58*, *OAS1*, *PARP9*, *DDX60*, *IFI35*, *IFI44*, *IFI44L*, *IRF9*, *XAF1* and *OAS2*.

Ultimately, 8 hub genes, *COL1A2*, *LOC100738989*, *MMP2*, *COL1A1*, *SPARC*, *POSTN*, *SERPINH1* and *FMOD* in the purple module were identified simultaneously using centiScape and cytoHubba. Function enrichment analysis showed that the 8 genes mainly involved in some pathways including AGE-RAGE signaling pathway in diabetic complications, Relaxin signaling pathway and Proteoglycans in cancer (Fig. 5d). The significant enriched BP terms were Extracellular matrix (ECM) organization and Extracellular structure organization, and so on (Fig. 5a). The significant enriched MF terms were Collagen binding and Extracellular matrix binding, and so on (Fig. 5b). The significant enriched CC terms were Extracellular matrix and Collagen-containing extracellular matrix, and so on (Fig. 5c).

In the magenta module, 19 hub genes, *USP18*, *MX1*, *STAT1*, *ISG15*, *IRG6*, *MX2*, *IFIT3*, *IFIT2*, *IFIT1*, *DDX58*, *OAS1*, *PARP9*, *DDX60*, *IFI35*, *IFI44*, *IFI44L*, *IRF9*, *XAF1* and *OAS2* were identified simultaneously using centiScape and cytoHubba. The functional enrichment analysis was performed for these hub genes. KEGG pathways had Hepatitis C, Coronavirus disease-COVID-19, Measles, Influenza A, Epstein-Barr virus infection, Herpes simplex virus 1 infection, NOD-like receptors signal pathway, RIG-I-like receptors signal pathway and Human papillomavirus infection and so on (Figure 6d). The significant enriched BP terms were Defense response to virus, Response to virus, Immune effector process, Defense response to other organism and Response to type I interferon (Fig. 6a). The enriched MF terms were Double-stranded RNA binding and GTP binding, and so on (Figure 6b). The enriched CC terms were Dendritic spine and Neuron spine, and so on (Fig. 6c).

Differential expression analysis

By the functional analysis and PPI network analysis, 18 genes and 24 genes were selected as key genes in the purple and magenta module, respectively. In the purple module, differential expression analysis showed that 17 genes except for *NPY1R* ($P=0.0502$) had significant differences in the purple module between two sex groups ($P<0.05$ or 0.01 , Fig. 7a and 7b). Among 17 genes with significant differences in expression, *FMOD*, *SPARC*, *POSTN*, *SERPINH1* and *LOC100522721* were significantly different ($0.01<P<0.05$), and other 12 genes were remarkable significant different ($P<0.01$). Expression level of *NPR1*, *LIPE*, *AQP7*, *PLA2G16* and *LOC100522721* in female pigs were significantly higher than that in male pigs, while that of *COL1A2*, *MMP2*, *COL1A1*, *SPARC*, *POSTN*, *SERPINH1*, *FMOD*, *LOC100738989*, *IGF1*, *FGF10*, *FGFR2* and *CACNA1G* in females were significantly lower than that in males. In the magenta module, 19 genes except for *USP18*, *MX1*, *IRG6*, *DDX60* and *ADAR* including *DDX58* ($P<0.05$), *OAS2* ($P<0.05$), *IRF9* ($P<0.05$), *STAT1* ($P<0.05$), *IFIT3* ($P<0.05$), *IFI44L* ($P<0.05$), *IFI44* ($P<0.05$), *IFI35* ($P<0.05$), *ISG15* ($P<0.05$), *MX2* ($P<0.05$), *PARP9* ($P<0.05$), *CXCL9* ($P<0.05$), *CXCL10* ($P<0.05$), *XAF1* ($P<0.05$), *IFIT1* ($P<0.01$), *IFIT2* ($P<0.01$), *OAS1* ($P<0.01$), *FAS* ($P<0.01$) and *TNFSF10* ($P<0.01$) were significantly differentially expressed between two sex groups, and all the genes were more highly expressed in female pigs (Fig. 7c and 7d).

Discussion

Key pathways

In our study, two modules including the purple and magenta modules closely related to sex in the subcutaneous fat tissue of pigs were identified using WGCNA method. Function enrichment analysis showed that the genes in the purple module were mainly enriched in some pathways including Regulation of lipolysis in adipocytes, Aldosterone synthesis and secretion, Ras signaling pathway and MAPK signaling pathway. These pathways are related to lipid metabolism [27, 28]. Yao et al. found that male gonadal steroids deficiency caused by castration significantly raised the serum lipid levels and increased fat accumulation in the pigs, and DEGs identified between the boars and barrows were mainly involved in metabolism of lipid, carbohydrate, amino acid and immune diseases pathways [29]. A study showed that male gonosteroids in boars could significantly reduce blood lipid levels and fat accumulation in pigs. Moreover, functional enrichment analysis showed that the downregulated genes in entire males as compared with immunocastrated males were significantly enriched in Adipocytokine signaling pathway, AMPK signaling pathway, Insulin signaling pathway and PPAR signaling pathway [30]. However, The genes in the magenta module were mainly involved in some pathways related to disease immunity, such as Influenza A, Hepatitis C, Measles, Herpes simplex infection, Defense response to virus and Response to virus, and so on. A study demonstrated that sex steroids also could regulate the immune system [31]. After hepatitis C virus and influenza A virus infection, inflammatory immune responses and repair of damaged tissue differed between the sex and impacted the outcome of infection [32, 33]. Therefore, it could be inferred that sex differences might affect fat deposition and immunity in subcutaneous fat tissue of pigs by affecting relevant pathways related to lipogenesis, lipid metabolism and disease immune.

Hub genes in the purple module

In the purple module, 8 hub genes including *COL1A2*, *MMP2*, *COL1A1*, *SPARC*, *POSTN*, *SERPINH1*, *FMOD* and *LOC100738989* were identified by the PPI analysis. Functional enrichment analysis showed that these genes were significantly enriched in AGE-RAGE signaling pathway in diabetic complications, Relaxin signaling pathway, Extracellular matrix (ECM) organization, Extracellular structure organization, ECM, Collagen-containing ECM, Collagen binding and ECM binding. The result was very similar to that from the study of Poklukur et al. [30], and their findings showed that the upregulated genes in entire males as compared with immunocastrated males and surgical castrates were significantly enriched in extracellular region/matrix (ECM) cellular components, ECM receptor interaction and focal adhesion pathways. Some genes responsible for the differences in backfat deposition between the three male sex categories were identified including *COL1A2*, *COL6A3*, *POSTN*, *P4HA3*, *DCN*, *FMOD*, *MMP2* and *MMP27* [30]. In our study, *COL1A2* gene was identified to have the highest degree in the PPI network, which indicated that the gene was at the core of the network. In the ECM remodeling, *COL1A2* and *COL1A1* genes involves in the synthesis of collagen, which are the major components of ECM [34]. *POSTN* gene is crucial for the collagen cross-linking and the ECM maintenance [35, 36]. Similarly, *FMOD* gene is required for proper collagen folding and ECM stabilization [37]. *SPARC* gene can promote the collagen fibrils development and post-translational modifications [38] and stabilizes the tissue and maintains the balance of lipogenesis and lipolysis [39]. *SERPINH1* gene is responsible for the correct folding and secretion of collagen [40], and can regulate the ECM proteins expression [41]. *MMP2* gene involves in the ECM degradation [42]. In the ECM remodeling, *COL1A2*, *COL1A1*, *FMOD*, *POSTN*, *SPARC*, *SERPINH1* and *MMP2* play a key role, which influence adipose tissue remodeling. In our study, the 7 genes were all significant differences in expression between different sexes, and expression of the 7 genes in boars were higher compared with sows. Jeong et al. measured the expression levels of ECM-related genes in different adipose tissues from bulls, cows and steers of Korean cattle (Hanwoo), and found that the expressions of ECM-related genes in the omental adipose tissue of cows and steers are decreased, and expression levels of most ECM-related genes were generally similar between cows and steers [43]. Poklukur et al. found that castration of male pigs resulted in the downregulation of genes involved in ECM dynamics [30]. The results of these studies are similar to this study. Thus, it might be inferred that sex differences could affect the expression levels of ECM-related genes (*COL1A2*, *COL1A1*, *SPARC*, *POSTN*, *FMOD*, *SERPINH1* and *MMP2*), progress to influence adipose tissue remodeling, and the higher expression of these genes in boars might make boars have a greater fat remodeling capacity than sows.

In the purple module, 10 hub genes including *LIPE*, *NPR1*, *NPY1R*, *AQP7*, *PLA2G16*, *LOC100522721*, *IGF1*, *FGF10*, *FGFR2* and *CACNA1G* were identified by the function enrichment analysis. Except for *NPY1R*, other genes were significant differences in expression between different sexes. Among these DEGs, the expression levels of *LIPE*, *NPR1*, *AQP7*, *PLA2G16* and *LOC100522721* in sows were significantly higher than that in boars, and other four genes including *LIPE*, *NPR1*, *AQP7* and *PLA2G16* excepting for *LOC100522721* participated in Regulation of lipolysis in adipocytes. *LIPE* gene encodes hormone sensitive lipase which cleaves free fatty acids from the intracellular triacylglycerol [44], and *LIPE* gene is significantly associated with intramuscular fat content and fatty acid composition of pigs [45, 46]. *NPR1* gene promotes the proliferation, differentiation and lipolysis of preadipocytes [47]. *AQP7* promotes glycerine excretion in adipose tissue and are highly expressed in adipose tissue [48], and *AQP7* deficiency is associated with adipose tissue triglyceride accumulation and adult obesity [49]. Similarly, *PLA2G16* is a major phospholipase A2 of adipose tissues that is specific to adipocytes [50]. *PLA2G16* encodes a major negative regulator of adipocyte lipolysis, and *PLA2G16* deficient mice has a markedly higher rate of lipolysis, and a significantly reduced adipose tissue mass [51, 52]. According to the above, *LIPE*, *NPR1*, *AQP7* and *PLA2G16* mainly involve in the regulation of lipolysis. At present, the four genes had not been reported in the study of fat deposition in pigs between different sexes.

Furthermore, among these DEGs, the expression levels of *IGF1*, *FGF10*, *FGFR2* and *CACNA1G* were significantly lower in sows than in boars. Functional enrichment analysis showed that *IGF1* participated in Ras signaling pathway, and *FGF10* and *FGFR2* participated in Ras signaling pathway and MAPK signaling pathway, while *CACNA1G* participated in Aldosterone synthesis and secretion and MAPK signaling pathway. A study showed that *IGF1* action is inhibited in castrated animals, which affects adipocyte proliferation and differentiation [30]. *FGF10*, belongs to the fibroblast growth factor family, which is widely involved in the regulation of cell growth, proliferation, differentiation and regulation of metabolism [53, 54]. *FGFR2* appears to be the most commonly distributed growth factor receptors and mediates most biological functions of the FGF family. Some studies suggested that *FGF10* stimulated preadipocyte proliferation and differentiation through activating *FGFR2* [55, 56]. Additionally, *CACNA1G* gene is reported to be associated with RFI, DMI and FCR [57, 58], not been reported in adipocyte proliferation and differentiation of pigs at present. As the above, *IGF1*, *FGF10* and *FGFR2* promote adipocyte proliferation and differentiation. Currently, the three genes had not been reported in the adipocyte proliferation and differentiation of pigs between different sexes. Because the expressions of the three genes were higher in boars, which indicated that the three genes might more promote adipocyte proliferation and differentiation of boars. In summary, *LIPE*, *NPR1*, *NPR1*, *PLA2G16*, *IGF1*, *FGF10* and *FGFR2* in the purple module were lipid metabolism-related genes, which might play a different regulatory role in the process of fat deposition and adipocyte proliferation and differentiation of pigs with different sexes.

Hub genes in the magenta module

In the magenta module, 19 genes were identified as hub genes by the PPI analysis. Functional enrichment analysis showed that these genes were mainly enriched in some diseases and signal pathways, such as Hepatitis C, Coronavirus disease-COVID-19, RIG-I-like receptors (RLRs) signal pathway and NOD-like receptors (NLRs) signal pathway, and so on. RLRs and NLRs can sense viral components, such as double/single-strand RNA and DNA. The RLRs play essential roles in the production of type I interferons (IFNs) and proinflammatory cytokines in cell type-specific manners [59]. Among these genes, 15 genes, *DDX58*, *STAT1*, *IRF9*, *OAS2*, *OAS1*, *IFIT1*, *IFIT2*, *IFIT3*, *ISG15*, *IFI35*, *IFI44*, *IFI44L*, *MX2*, *XAF1* and *PARP9* were significant differences in subcutaneous adipose tissue of pigs between two sex groups. It has been reported that *DDX58* gene belongs to one of the crucial members of RLRs family, and *DDX58* can produce type I interferon (IFN) [60, 61], which activates *STAT1* and *STAT2* and recruits *IRF9* gene. The *STAT1*-*STAT2*-*IRF9* complex activates IFN-stimulated gene factor 3 (*ISGF3*), which drives the expression of IFN-stimulated genes (ISGs) [62, 63]. A study showed that *STAT1* and *IRF9* were the transcription factors (TFs) for interferon and played important roles in the immune system [64]. Some studies showed that *OAS2* [65], *OAS1* [65], *IFIT1* [66], *IFIT2* [67], *IFIT3* [67], *ISG15* [68], *IFI35* [69], *IFI44* [70], *IFI44L* [70], *PARP9* [70], *XAF1* [71] and *MX2* [72] belonged to the Type I ISGs. *OAS1* and *OAS2* are the 2'-5' oligoadenylate synthetase family which consists of antiviral enzymes induced by IFN and is responsible for destroying virus-derived dsRNA [65]. *IFIT1*, *IFIT2* and *IFIT3* belong to interferon induced proteins with tetratricopeptide repeats family, which have a great importance in antiviral and immune regulation [67] and restrict virus infection through altering cellular protein synthesis [73]. *ISG15*, *IFI35*, *IFI44* and *IFI44L* belong to IFN-induced protein family. *ISG15* gene is one of the most abundantly induced ISGs and can effectively control certain viral and bacterial infections [74]. A study indicated that *IFI35*, *IFI44* and *IFI44L* played important roles in interferon signaling, anti-bacterial and immune regulation [75]. Beside, *PARP9* gene promotes the expression of pro-inflammatory genes [76]. *XAF1* gene involves in immunoregulatory [71]. *MX2* gene mediates antiviral innate immunity [77].

In the magenta module, 12 genes were identified as key genes by the function enrichment analysis. Among these genes, 10 genes, *DDX58*, *STAT1*, *IRF9*, *OAS2*, *OAS1*, *IFIT1*, *CXCL9*, *CXCL10*, *TNFSF10*/TRAIL and *FAS* were significant differences between two sex groups. The six genes, *DDX58*, *STAT1*, *IRF9*, *OAS2*, *OAS1* and *IFIT1* have been discussed before. Some studies showed that *CXCL9* [78], *CXCL10* [79] and *TNFSF10*/TRAIL [70] were also Type I ISGs. The proinflammatory chemokines *CXCL9* and *CXCL10* associate with inflammation and proliferation [80, 81]. TRAIL-system plays a role in regulating autoimmunity [82]. *FAS* gene involves in activation of the Type I IFN/STAT1 axis [83]. From the above, a total of 19 DEGs in the magenta module participated in Type I IFN response, and it could be inferred that *DDX58* gene first promoted the production of type I IFN, while *FAS* gene participated in activating the Type I IFN/STAT1 axis. Second, the combination of *STAT1*, *STAT2* with *IRF9*, which drove the expression of 15 ISGs (*OAS2*, *OAS1*, *IFIT1*, *IFIT2*, *IFIT3*, *ISG15*, *IFI35*, *IFI44*, *IFI44L*, *PARP9*, *XAF1*, *MX2*, *CXCL9*, *CXCL10* and *TNFSF10*) which encoded corresponding immune proteins to carry on specific immune action. Currently, the 17 genes and two TFs (*STAT1* and *IRF9*) had not been reported in the disease immunity of pigs between different sexes. In this study, the expressions of these genes were higher in female pigs. Therefore, the 17 genes, *DDX58*, *FAS*, *OAS2*, *OAS1*, *IFIT1*, *IFIT2*, *IFIT3*, *ISG15*, *IFI35*, *IFI44*, *IFI44L*, *PARP9*, *XAF1*, *MX2*, *CXCL9*, *CXCL10* and *TNFSF10*) and two TFs (*STAT1* and *IRF9*) participated in Type I IFN response which might play a different regulatory role in immunity of pigs between different sexes and might enhance the immunity ability of female pigs though Type I IFN stimulated pathway in cells.

However, there were some limitations in this study. On one hand, small sample size limited the statistical power to identify the hub genes. On the other hand, molecular biological experiments were required to validate the function of these hub genes.

Conclusions

In summary, 16 coexpression modules were identified using WGCNA method, and two of these modules were closely related to sex. Functional enrichment analysis showed that the purple module was related to lipid metabolism, while the magenta module was associated with immunity. 7 ECM-related genes (*COL1A2*, *COL1A1*, *SPARC*, *POSTN*, *FMOD*, *SERPINH1* and *MMP2*) and 7 genes related to lipid metabolism (*LIPE*, *AQP7*, *NPR1*, *PLA2G16*, *IGF1*, *FGF10* and *FGFR2*) were identified in the purple module. In the magenta module, 17 genes and two TFs participating in Type I IFN response were identified which were associated with immunity. In conclusion, the identified key pathways and genes in subcutaneous adipose tissue from different sexes of pigs, which provided some insights into the molecular mechanism involving in fat formation and immunoregulation between male and female pigs. These findings may be helpful for breeding in pig industry and obesity treatment in medicine.

Methods

RNA-seq data and samples information collection

The normalized gene expression dataset of GSE61271 was used in this study, and was retrieved from the public NCBI GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). The GSE61271 dataset was from a study of Kogelman et al. [84], consisting of 36 pig (crossbred F2 of Duroc and Göttingen miniature pig) samples with 100 kg weight (17 females and 19 males).

WGCNA

WGCNA was used to construct the gene coexpression network, and identify the coexpression gene modules. The WGCNA package (version 1.13) based on R was used to perform WGCNA [13]. First, the expression matrix was converted into an adjacency matrix, and an unsupervised coexpression relationship was constructed based on the adjacency matrix using Pearson correlation coefficients for gene pairs. The correlation adjacency matrix was strengthened by power β (soft threshold), and the power parameter was selected based on scale-free topology criterion.

Second, the adjacency matrix was transformed into a topology matrix, and TOM was used to measure the correlation of gene pairs. According to 1-TOM, average linkage hierarchical clustering was performed to classify genes with coherent expression profiles into gene modules. Dynamic cutting algorithm was used to identify gene modules from the system cluster tree. Module eigengene (ME) was defined as the first principal components, and was the representative in module genes. Module membership (MM) was defined as the correlation between ME and gene module. Gene significance (GS) was indexed by $\log_{10}$ transformation of the P value of T-test. The only requirement was that GS of 0 indicates that the gene was not significant with regard to the biological question of interest. The GS could take on positive or negative values. Module significance (MS) was defined as the average GS for all the genes in the module. More detailed description of WGCNA was presented by an original article [13].

Finally, the statistical significance of the relationship between modules and sexes was analyzed by calculating the Pearson correlation coefficient. For studying the genes in the module related to sex, the top two modules with the high GS scores among all modules were selected as the modules of significant for further analysis.

PPI network construction and analysis

The interactive relationships among genes encoding proteins in the significant gene coexpression module were analyzed by constructing a PPI network. The interactive information among genes encoding proteins was retrieved from online the Search Tool for the Retrieval of Interacting Genes (STRING) database (version 11.5, <https://string-db.org/>). The gene pairs with a combined score $\geq$ the medium confidence 0.4 were used to construct the PPI network. Cytoscape (v3.8.0) software was used to construct and visualize the interactive relationships among genes in whole PPI network [85].

GO and KEGG analysis

GO and KEGG pathway terms of all genes in the significant module were analyzed using the online DAVID database (version 6.80, <https://david.ncifcrf.gov/>). The cut-off criterion was set as $P\text{-value} < 0.05$. Cytoscape (v3.8.0) software was used to construct and visualize the interactive relationships between genes and functional enrichment terms in the whole network. GO and KEGG enrichment analyses for hub genes in PPI network were implemented using the R-package clusterProfiler software [86]. The cut-off criterion was set as $q\text{-value} < 0.5$. GO annotation result includes three main bodies: biological process (BP), molecular function (MF) and cellular component (CC).

Hub genes identification

Hub genes were identified by the cytoHubba algorithm and centiScape 2.2 plugin in the cytoscape software, and key genes were identified using GO and KEGG network analysis. CytoHubba algorithm was used to gain top genes in the whole PPI network, and the criterion of selecting hub genes was that top nodes ranked by Maximal Clique Centrality (MCC) [87]. The centiScape algorithm was applied to calculate the centrality of each gene in the whole PPI network, including Degree Centrality, Betweenness Centrality, Closeness Centrality, EigenVector Centrality, Stress Centrality and Radiality Centrality, and so on [88]. The genes with higher Centrality scores were identified as hub genes.

Statistical analysis

All statistical analyses were performed using R software. The comparison of gene expression in subcutaneous fat tissues with females and male pigs was analyzed using Mean $\pm$ Standard Deviation. T-test was used to estimate the statistical difference, and $0.01 < P < 0.05$ was considered as significant difference, and $P < 0.01$ was considered as remarkable significant different, and $P > 0.05$ was considered as not significant difference in expression level between two groups.

Abbreviations

WGCNA: Weighted gene coexpression network analysis; PPI: Protein and Protein Interaction; GEO: Gene Expression Omnibus; DAVID: Database for Annotation, Visualization and Integrated Discovery; STRING: Search Tool for the Retrieval of Interacting Genes; BP: Biological process; CC: Cellular component; MF: Molecular function; DEGs: Differential expression genes; TOM: Topological overlap measure; 1-TOM: TOM-based dissimilarity; ME: Module eigengene; GS: Gene significance; MM: Module membership; MS: Module significance; MCC: Maximal clique centrality; ECM: Extracellular matrix; IFN: Interferon; ISG: IFN-stimulated gene; RLR: RIG-I-like receptor; NLR: NOD-like receptor; TF: Transcription factor

Declarations

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Not applicable.

Authors' contributions

HYW, XYW, SXL and QC processed and analyzed the data. MLL and SYW assisted with the processing of data. HYW and XYW wrote the manuscript that was subsequently revised by SXL and QC. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets analysed during the current study are available in the public NCBI GEO database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61271>) [84].

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Tables

Table 1

The results of GO and KEGG enrichment analysis for the purple module using DAVID tool

ID	KEGG/GO term	Gene symbols
KEGG		
ssc04923	Regulation of lipolysis in adipocytes	<i>NPR1</i> <i>LIPE</i> <i>AQP7</i> <i>PLA2G16</i> <i>NPY1R</i>
ssc04925	Aldosterone synthesis and secretion	<i>NPR1</i> <i>LIPE</i> <i>CACNA1G</i> <i>PLCB4</i> <i>CREB3L1</i>
ssc04014	Ras signaling pathway	<i>FGFR2</i> <i>FGF10</i> <i>IGF1</i> <i>PLA2G16</i> <i>FOXO4</i> <i>PLA1A</i> <i>LOC100522721</i>
ssc04010	MAPK signaling pathway	<i>FGFR2</i> <i>FGF10</i> <i>DDIT3</i> <i>CACNA1G</i> <i>GADD45G</i> <i>LOC100620270</i> <i>LOC100522721</i>
Biological process		
GO:0048644	Muscle organ morphogenesis	<i>CNTF</i> <i>GSC</i> <i>ARID5B</i>
Cellular component		
GO:0005615	Extracellular space	<i>FGF10</i> <i>IGF1</i> <i>MMP2</i> <i>CHRD</i> <i>SMPDL3B</i> <i>VASN</i> <i>CNTF</i> <i>SLIT2</i> <i>CPXM1</i> <i>RAB11FIP4</i> <i>LOC100739844</i> <i>JAM3</i> <i>C1QTNF6</i> <i>KERA</i> <i>AE</i>
Molecular function		
GO:0008201	Heparin binding	<i>FGF10</i> <i>CHRD</i> <i>FMOD</i> <i>SLIT2</i> <i>POSTN</i> <i>PCOLCE</i> <i>CCDC80</i>

Table 2

The results of GO and KEGG enrichment analysis for the magenta module using DAVID tool

Table 3

Top 10 genes with higher scores identified by centrality analyses using centiScape in the purple module

ID	KEGG/GO term	Gene symbols	P value
KEGG			
ssc05164	Influenza A	<i>FAS</i> ; <i>STAT1</i> ; <i>DDX58</i> ; <i>OAS2</i> ; <i>OAS1</i> ; <i>MX1</i> ; <i>IRF9</i> ; <i>CXCL10</i> ; <i>TNFSF10</i> ; <i>ADAR</i> ; <i>RSAD2</i> ; <i>LOC100157336</i>	1.01e-06
ssc05162	Measles	<i>FAS</i> ; <i>STAT1</i> ; <i>DDX58</i> ; <i>OAS2</i> ; <i>OAS1</i> ; <i>MX1</i> ; <i>IRF9</i> ; <i>TNFSF10</i> ; <i>ADAR</i> ; <i>IL2RB</i>	8.02e-06
ssc05168	Herpes simplex infection	<i>FAS</i> ; <i>STAT1</i> ; <i>DDX58</i> ; <i>OAS2</i> ; <i>OAS1</i> ; <i>IRF9</i> ; <i>IFIT1</i> ; <i>LOC100157336</i> ; <i>TAP2</i> ; <i>IL15</i> ; <i>SLA-1</i>	1.65e-05
ssc05160	Hepatitis C	<i>STAT1</i> ; <i>DDX58</i> ; <i>OAS2</i> ; <i>OAS1</i> ; <i>IRF9</i> ; <i>IFIT1</i> ; <i>IRF1</i> ; <i>LDLR</i>	3.30e-04
Biological process			
GO:0051607	Defense response to virus	<i>CXCL9</i> ; <i>CXCL10</i> ; <i>MX1</i> ; <i>OAS2</i> ; <i>OAS1</i> ; <i>IRF1</i> ; <i>IFIT1</i> ; <i>IFIT2</i> ; <i>IFIT3</i> ; <i>ISG15</i> ; <i>LOC733579</i> ; <i>CD8A</i> ; <i>RSAD2</i> ; <i>PRF1</i> ; <i>ADAR</i>	7.95e-12
GO:0006955	Immune response	<i>FAS</i> ; <i>CXCL9</i> ; <i>CXCL10</i> ; <i>OAS2</i> ; <i>OAS1</i> ; <i>IL15</i> ; <i>CD244</i> ; <i>LOC106504143</i> ; <i>CTSW</i> ; <i>TNFSF10</i> ; <i>SMAD6</i> ; <i>LTB</i> ; <i>SLA-1</i> ; <i>LOC100513601</i>	8.50e-08
Cellular component			
GO:0009897	External side of plasma membrane	<i>FAS</i> ; <i>CXCL9</i> ; <i>CXCL10</i> ; <i>CD8A</i> ; <i>CD244</i> ; <i>LDLR</i> ; <i>CD2</i> ; <i>KLRK1</i>	1.77e-04
Molecular function			
GO:0003725	Double-stranded RNA binding	<i>DDX58</i> ; <i>OAS2</i> ; <i>OAS1</i> ; <i>DHX58</i>	0.0076295

Gene	Degree	Betweenness	Closeness	EigenVector	Stress	Radiality
<i>COL1A2</i>	19	837.8553114	0.005747126	0.363597997	2948	4.8
<i>LOC100738989</i>	16	563.4065018	0.005649718	0.329498621	2276	4.773913043
<i>MMP2</i>	15	889.0868132	0.005882353	0.318458854	3766	4.834782609
<i>COL1A1</i>	13	161.1777473	0.005	0.289918992	1024	4.573913043
<i>SPARC</i>	12	45.19917582	0.004878049	0.3010476	334	4.530434783
<i>POSTN</i>	11	215.0187729	0.004975124	0.268061831	1462	4.565217391
<i>IGF1</i>	10	2121.740842	0.00617284	0.124305981	4578	4.904347826
<i>SERPINH1</i>	10	396.5845238	0.00456621	0.214246397	1790	4.408695652
<i>FMOD</i>	9	270.4775641	0.004807692	0.215407852	832	4.504347826
<i>FKBP10</i>	8	464.2095238	0.004504505	0.166622554	2116	4.382608696

Table 4

Top 20 genes with higher scores identified by centrality analyses using centiScape in the magenta module

Gene	Degree	Betweenness	Closeness	EigenVector	Stress	Radiality
<i>USP18</i>	49	1035.767393	0.003861004	0.214286633	8528	6.717105263
<i>MX1</i>	47	1467.536254	0.003891051	0.210942217	9596	6.730263158
<i>STAT1</i>	46	1597.399356	0.003937008	0.199111377	14050	6.75
<i>ISG15</i>	44	646.8937011	0.003745318	0.207317681	5516	6.664473684
<i>IRG6</i>	43	394.756316	0.003745318	0.205145769	5600	6.664473684
<i>MX2</i>	40	1425.201521	0.003717472	0.191899951	9226	6.651315789
<i>IFIT3</i>	39	195.9297022	0.003636364	0.196834362	3234	6.611842105
<i>IFIT2</i>	38	172.7290035	0.003496503	0.189962271	2596	6.539473684
<i>IFIT1</i>	38	1115.323993	0.003597122	0.183495334	7916	6.592105263
<i>DDX58</i>	38	382.6723592	0.003649635	0.187862494	4164	6.618421053
<i>OAS1</i>	37	748.0567294	0.003484321	0.181182841	4892	6.532894737
<i>PARP9</i>	32	135.1189157	0.003436426	0.166187174	2012	6.506578947
<i>DDX60</i>	32	752.0138271	0.003355705	0.164950398	6274	6.460526316
<i>IFI44L</i>	31	65.3413002	0.003333333	0.169057372	1230	6.447368421
<i>IFI35</i>	31	109.2484837	0.003367003	0.161532744	1644	6.467105263
<i>IFI44</i>	31	51.3264405	0.003355705	0.171500135	1016	6.460526316
<i>PARP14</i>	29	86.78062865	0.00330033	0.156260384	1400	6.427631579
<i>IRF9</i>	28	288.8620542	0.00330033	0.153975259	2196	6.427631579
<i>XAF1</i>	26	59.40043809	0.003322259	0.145687373	976	6.440789474
<i>OAS2</i>	26	17.67701199	0.003246753	0.149119245	438	6.394736842

Figures

Figure 1

WGCNA analysis. (a) Scale independence and mean connectivity of various soft-thresholding values (b). The left panel (a) displayed the influence of soft-thresholding power (X-axis) on scale-free fit index (Y-axis). The right panel (a) showed the influence of soft-thresholding power (X-axis) on the mean connectivity (degree, Y-axis). (b) Cluster Dendrogram of all filtered genes enriched based on the dissimilarity measure and the cluster module colors. (c) Heatmap plot of the adjacencies of modules. Red represented high adjacency (positive correlation) and blue represented low adjacency (negative correlation). (d) Network heatmap plot of all genes. (e) Matrix with the Module-Trait Relationships (MTRs) and corresponding P-values between the detected modules on the y-axis and sex on the x-axis. (f) Gene significance was defined as the mean of the absolute value of the correlation coefficient for all genes with 16 modules. The higher mean gene significance represented the higher correlation between module and sex. WGCNA, weighted gene co-expression network analysis.

Figure 2

Gene significance vs. Module membership and Gene expression heatmap for the significant module. Association between the module membership and gene significance within the purple module (a) and the magenta module (b). Heatmap of expression of all genes in the purple module (c) and the magenta module (d). Female (nattier blue) represented female pigs, and Male (pink) represented male pigs. X-axis represented the samples. Y-axis represented genes in the module, and blue represented down-regulated genes and red represented up-regulated genes. The color scale showed the expression values.

Figure 3

Function enrichment network for the significant module. (a) 4 KEGG pathways, 3 GO terms and 41 genes were used to construct pathway-gene network for the purple module. (b) 4 KEGG pathways, 4 GO terms and 35 genes were used to construct pathway-gene network for the magenta module. Blue triangle represented KEGG pathway, and blue diamond represented GO terms. Circle represented genes, and green and blue circle represented selected genes and red circle represented unselected genes.

Figure 4

Protein-protein interaction (PPI) network for the significant module. (a) PPI network for the purple module. There were 116 nodes and 169 edges in the network. (b) PPI network for the magenta module. There were 153 nodes and 814 edges in the network. These nodes (circles) represented genes and edges (gray lines) between nodes indicated the interaction of genes in the network. Size of circles represented degree number of node, and the bigger the size, the more degree the node. Color represented betweenness, and the darker the color, the more betweenness the node. (c) PPI sub-network for the purple module. There were 10 nodes and 36 edges in the first sub-network. (d) PPI sub-network for the magenta module. There were 20 nodes and 187 edges in the second sub-network. Size of circles represented degree number of node, and the bigger the size, the more degree the node. Color represented rank, and the darker the color, the more rank the node.

Figure 5

KEGG and GO enrichment analysis for 8 hub genes were identified using two cytoscape plugins in the first PPI network. GO including (a) Biological process (BP), (b) Molecular function (MF) and (c) Cellular component (CC) and (d) KEGG enrichment analysis. Top 5 terms ordered by P.adjust. P.adjust indicated the degree of enrichment, with smaller P.adjust indicating terms that were more likely to play significant functional roles.

Figure 6

KEGG and GO enrichment analysis for 19 hub genes were identified using two cytoscape plugins in the second PPI network. GO including (a) Biological process (BP), (b) Molecular function (MF) and (c) Cellular component (CC) and (d) KEGG enrichment analysis. Top 5 terms ordered by P.adjust. P.adjust indicated the degree of enrichment, with smaller P.adjust indicating terms that were more likely to play significant functional roles. Top 10 terms ordered by P.adjust for the KEGG enrichment analysis.

Figure 7

The expression boxplot of key genes in the purple and magenta modules. (a) 8 key genes, and (b) 10 key genes in the purple module. (c) 12 key genes, and (d) 12 key genes in the magenta module. 0.01<P<0.05 was considered as significant difference, and P<0.01 was considered as remarkable significant different, and P>0.01 was considered as not significant difference between two groups in expression level. X-axis represented the genes, and Y-axis represented value of gene expression. F (red) represented female pigs, m (blue) represented male pigs.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1Thenumberofgenesineachofthe16modules.docx](#)