Identification of key genes and pathways associated with obesity in children

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Abstract. The present study aimed to identify potential key genes and pathways in obese children in order to explore possible molecular mechanisms associated with child obesity. The array dataset GSE29718 was downloaded from the Gene Expression Omnibus database. Subcutaneous adipose tissue samples derived from 7 obese children and 8 lean children were selected for the analysis. Differentially expressed genes (DEGs) in samples from obese children compared with those from lean children were analyzed by the limma package. Gene ontology (GO) annotation, Kyoto Encyclopedia of Genes and Genomes and Reactome pathway enrichment analyses for up and downregulated genes were performed. A protein-protein interaction (PPI) network was constructed with Cytoscape software and important genes associated with obesity were determined using IRegulon. A total of 199 DEGs (79 up and 120 downregulated genes) were identified in the samples of obese children compared with those from lean children. The PPI network was established with 103 nodes and 147 protein pairs. Matrix metalloproteinase 9 (MMP9) and acetyl-CoA carboxylase β (ACACB) were identified as hub genes in the PPI network and may therefore be marker genes for child obesity. In addition, upregulated DEGs were enriched in Reactome pathways associated with the immune system. Besides, MMP9 was upregulated in immune system processes as a GO term in the category Biological Processes. The results of the present study indicated that MMP9, ACACB and immune system pathways may have a significant role in child obesity.

Introduction

Obesity is a medical condition in which excess body fat has accumulated to a certain degree, and affected individuals may have a reduced life expectancy and increased health problems (1). Child obesity is becoming a health problem in developed and developing countries (2,3). Obesity is caused by a combination of genetic, behavioral, social, cultural, metabolic and physiological factors (4). Child obesity increases the likelihood of certain diseases, such as hyperlipidemia, insulin resistance and hypertension (5). The physiological mechanisms associated with obesity have been investigated in-depth (6), but certain key molecular mechanisms involved in obesity have remained to be identified.

Previous studies have determined certain pathological mechanisms associated with obesity. One study indicated that body fat and weight were inversely associated with 25-hydroxyvitamin D levels and volumetric dilution may explain for the low vitamin D levels in obese individuals (7). It also has been suggested that a high-fat diet accelerated obesity via the Toll-like receptor 4 signaling pathway (8). In addition, leptin-melanocortin signaling is a key pathway in regulating food intake and body weight, and mutation of ligands or receptors in this pathway may cause obesity (9). Iodothyronine deiodinase type 2 has a role in the progression of obesity via the c-Jun N-terminal kinase (JNK) signaling pathway (10). Activation of the adenosine monophosphate-activated protein kinase signaling pathway has been reported to prevent obesity (11). Furthermore, Vohl et al (12) reported that 216 and 131 genes were overexpressed in visceral fat and subcutaneous adipose tissues, respectively. Linder et al (13) reported that several genes, including the phospholipid transfer gene, ras, adipsin and calcyclin were differentially expressed in adipose tissue of male or female patients. In addition, two previous studies indicated that variation in the fat mass and obesity-associated gene was associated with childhood obesity and severe adult obesity (14,15). However, to the best of our knowledge, few articles have reported the genes or pathways of

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childhood obesity alone. Therefore, further significant genes or pathways associated with child obesity are required to be identified.

The microarray data of GSE29718 have been previously used to reveal the links between type 2 diabetes and obesity (16) or used to detect molecular mechanisms for the association between obesity and colorectal cancer (17). The present study identified the differentially expressed genes (DEGs) in obese children compared with those in lean children based on the microarray data of GSE29718. Functional enrichment analyses of DEGs were then performed. In addition, a protein-protein interaction (PPI) network was established and important genes associated with obesity were analyzed. The present study aimed to identify critical genes or pathways associated with child obesity and explored possible underlying molecular mechanisms.

Materials and methods

Affymetrix microarray data. The array dataset GSE29718 deposited by Tam *et al* (18) was downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). The dataset contained 5 visceral adipose tissue samples and 15 subcutaneous adipose tissue samples from children. Only the subcutaneous adipose tissue samples derived from 7 obese and 8 lean children were used for the subsequently analysis. The array data were based on the GPL6244 Affymetrix Human Gene 1.0 ST Array platform, transcript (gene) version (Affymetrix Inc., Santa Clara, CA, USA).

Data pre-processing. The raw data were pre-processed by the robust multiarray average (19) algorithm with the use of oligo (20) in the R software of Bioconductor (Seattle, Washington). The process of pre-processing included background correction, normalization and calculation of gene expression. Finally, a total of 18,977 gene expression values was obtained.

DEG analysis. The DEGs in samples from obese children compared with those from lean children were analyzed by the limma package (21) in Bioconductor. The P-values of the DEGs were calculated by using the unpaired Student's t-test (22) in the limma package in the process of analysis. $|\log_2 FC| \geq 0.4$ and P<0.05 were set as cut-off criteria.

Gene ontology (GO) and pathway enrichment analyses. GO includes 3 categories, namely molecular function, biological processes (BP) and cellular components, and is a tool used for gene annotation by using a defined, structured and controlled vocabulary (23). The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database used to assign sets of DEGs to specific pathways (24). Reactome is a database used for forging a link between metabolome and genome (25).

Up and downregulated genes were subjected to GO annotation as well as KEGG and Reactome pathway enrichment analyses. P<0.05 was used as the threshold value, and the number of genes enriched in each pathway was ≥2.

PPI network analysis. The Search Tool for the Retrieval of Interacting Genes (STRING) (26) can be used to provide information regarding predicted and experimental interactions of proteins and the prediction method of this database is from neighborhood, gene fusion, co-occurrence, co-expression experiments, databases and textmining. The DEGs were mapped into PPIs and a combined score of >0.4 was set as a threshold value in this study. PPI networks were constructed with Cytoscape software (27). Moreover, the nodes with higher degrees of interaction were considered as hub nodes.

The association among nodes with a higher degree of interaction in the network and the associated biological functions were analyzed by literature mining. GenCLiP 2.0 (28) (http://ci.smu.edu.cn/GenCLiP2.0/confirm_keywords.php) is an online tool used for the literature mining analysis of human genes and networks. Hub gene sets obtained from the PPI network were used as input gene sets. Based on the user-defined query terms, the Literature Mining Gene Networks module can construct gene networks, generate sub-networks and calculate the likelihood of the networks' random occurrence using random simulation (29). In order to annotate the input genes, Gene Cluster with Literature Profiles module can generate statistically over-represented keywords. The keywords grouped by a fuzzy algorithm can be provided by the user or are generated according to occurrence of free terms in the literature on the respective gene. The relevant Medline abstracts (co-occurrence of genes and keywords are highlighted) are linked via the associations between genes and keywords. The co-citation network of key genes in the literature was analyzed using the 'Literature Mining Gene Networks' module in GenCLiP to analyze hot associated genes in a literature research. Subsequently, the associated biological function of these hot genes by 'Gene Cluster with Literature Profiles' modules with $P \leq 1 \times 10^{-6}$ and hit ≥6.

Analyses of important genes and transcription factors associated with obesity. The comparative toxicogenomics database (CTD) (30) is a tool used to formalize, harmonize and centralize data of genes and proteins across different species. The present study assessed whether any of the marker genes among the DEGs identified had been previously listed as markers of obesity in the CTD database. 'Obesity' was used as an input key word in CTD. Subsequently, the cytoscape plugin iRegulon (31) was used to analyze transcription factors regulating marker genes. iRegulon uses cis-regulatory sequence analysis to reverse-engineer the transcriptional regulatory network underlying a co-expressed gene set. It integrates the transcription factor information from databases such as Transfac, Jaspar, Encode, Swissregulon and Homer, and detects enriched transcription factor motifs and optimal sets of their direct targets by means of genome-wide ranking and recovery. Parameter settings were as follows: Minimum identity between orthologous genes=0.05 and maximum false discovery rate on motif similarity=0.001. The Normalized Enrichment Score (NES) was the output result. The higher the scores were, the more reliable the results were. Transcription factors and target gene pairs with NES>5 were selected.

Results

DEG analysis. As shown in Fig. 1, a total of 199 DEGs (79 up and 120 downregulated genes) were identified in samples from
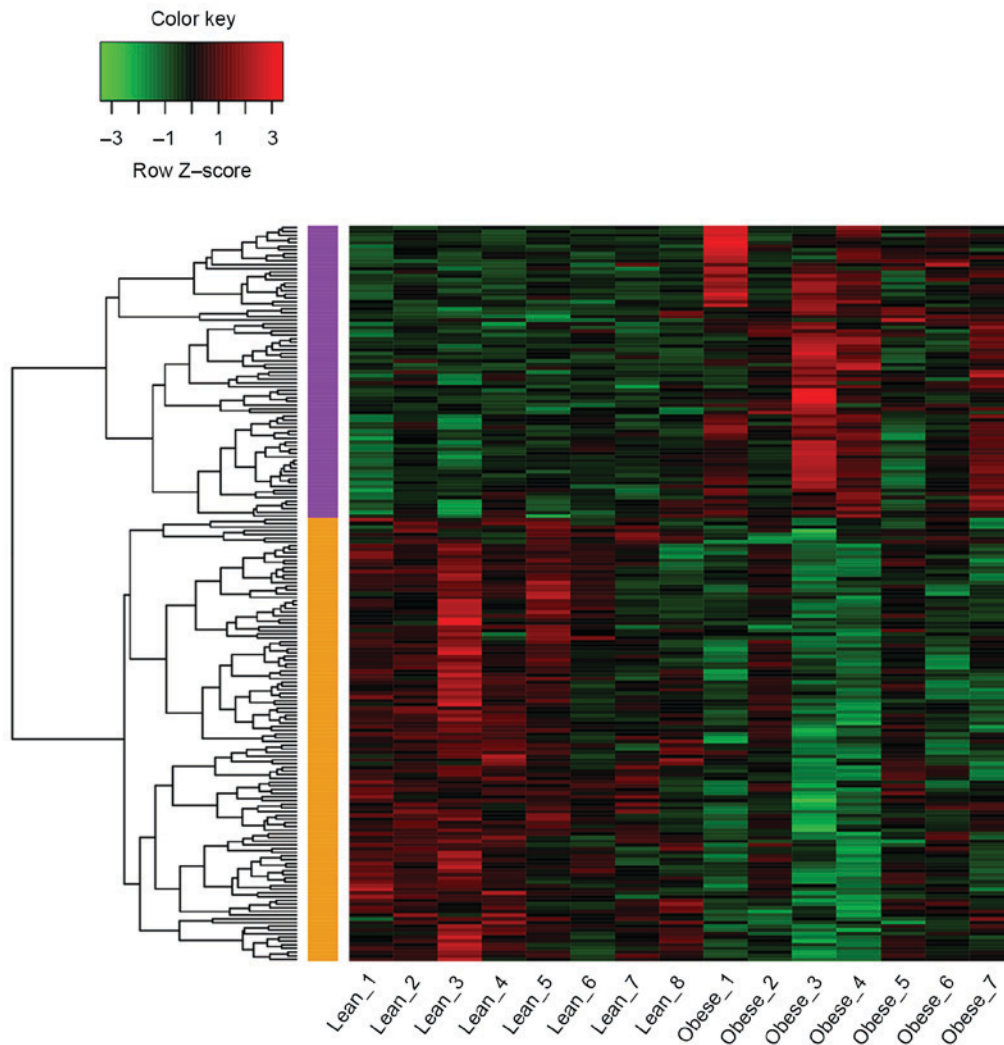
Figure 1. Heat map of differentially expressed genes. Green colour represents a lower and red colour represents a higher expression level. Black colour indicates no differential expression.

obese children compared with those from lean children with P<0.05 and |log$_2$FC|≥0.4. The average log$_2$FC of upregulated genes was 0.585 and that of downregulated genes was -0.558.

GO and pathway enrichment analyses. GO, KEGG pathway and Reactome pathway analyses were performed for up and downregulated DEGs. Upregulated DEGs were mainly enriched in the GO terms of extracellular space, immune response and immune system process (Table I). In addition, matrix metalloproteinases 9 (MMP9) was significantly enriched in pathways of immune system processes. The downregulated DEGs were mainly associated with the regulation of system process and cyclic guanosine monophosphate (cGMP)-inhibited cyclic nucleotide (Table I).

The significantly enriched KEGG pathways of upregulated DEGs were cell adhesion molecules and phagosome (Table IIA). The significantly enriched KEGG pathways of downregulated DEGs were nitrogen metabolism and propanoate (Table IIA). The significantly enriched Reactome pathways of upregulated DEGs were immune system and adaptive immune system (Table IIB). The significantly enriched Reactome pathways by downregulated DEGs were signaling by retinoic acid and cGMP effects (Table IIB).

PPI network analysis. A total of 103 nodes and 147 protein pairs were obtained with a PPI score of >0.4 based on the STRING database (Fig. 2). The interactions of proteins encoded by the DEGs were inconspicuous. The proteins that were closely associated with other proteins with a degree of interaction ≥10 were MMP9 (degree=16), Acetyl-CoA carboxylase β (ACACB; degree=13), MET proto-oncogene, receptor tyrosine dinase (MET; degree=11) and von Willebrand factor (VWF; degree=10). Literature mining was performed for 8 genes with high degree of interaction in the networks. The co-citation network of these 8 genes in the reported literature is shown in Fig. 3A and the results reported by previous studies regarding these genes are shown in Table III. Significant results of enrichment analysis of these 8 genes reported the literature are shown in Fig. 3B, and enrichment was identified in terms such as binding protein 1, reactive oxygen species and kinase activity.

Analysis of marker genes and transcription factors associated with obesity. A total of 11 previously reported marker genes of obesity listed in the CTD, including ACACB, MMP9, glutamate-ammonia ligase and ferritin light polypeptide, were identified among the DEGs of the present study. The

Table I. GO analysis of differentially expressed genes (P<0.05).

GO ID	Term	No. of genes	P-value
Upregulated genes			
GO-BP terms			
GO:0006955	Immune response	25	2.25×10^{-10}
GO:0002376	Immune system process	31	6.67×10^{-10}
GO:0006954	Inflammatory response	15	1.57×10^{-8}
GO:0009605	Response to external stimulus	27	2.20×10^{-8}
GO:0006952	Defense response	23	2.41×10^{-8}
GO-CC terms			
GO:0005615	Extracellular space	21	1.26×10^{-8}
GO:0005576	Extracellular region	36	4.13×10^{-6}
GO:0044421	Extracellular region part	32	5.06×10^{-6}
GO:0005578	Proteinaceous extracellular matrix	7	4.62×10^{-4}
GO:0005886	Plasma membrane	32	9.04×10^{-4}
GO-MF terms			
GO:0043394	Proteoglycan binding	3	1.83×10^{-4}
GO:0001948	Glycoprotein binding	4	3.47×10^{-4}
GO:0008061	Chitin binding	2	4.62×10^{-4}
GO:0043395	Heparan sulfate proteoglycan binding	2	1.94×10^{-3}
GO:0030246	Carbohydrate binding	5	5.04×10^{-3}
Downregulated genes			
GO-BP terms			
GO:0044057	Regulation of system process	16	4.50×10^{-9}
GO:0008015	Blood circulation	15	3.72×10^{-8}
GO:0003013	Circulatory system process	15	3.97×10^{-8}
GO:0051239	Regulation of multicellular organismal process	36	5.25×10^{-8}
GO:2000021	Regulation of ion homeostasis	10	1.20×10^{-7}
GO-CC terms			
GO:0031093	Platelet alpha granule lumen	4	1.00×10^{-4}
GO:0031091	Platelet alpha granule	4	6.00×10^{-4}
GO:0034774	Secretory granule lumen	4	6.00×10^{-4}
GO:0009925	Basal plasma membrane	3	7.00×10^{-4}
GO:0060205	Cytoplasmic membrane-bounded vesicle lumen	4	1.40×10^{-3}
GO-MF terms			
GO:0004119	cGMP-inhibited cyclic nucleotide phosphodiesterase activity	2	3.65×10^{-5}
GO:0043168	Anion binding	31	1.00×10^{-4}
GO:0004740	Pyruvate dehydrogenase (acetyl-transferring) kinase activity	2	2.00×10^{-4}
GO:0004114	3',5'-Cyclic nucleotide phosphodiesterase activity	3	4.00×10^{-4}
GO:0097367	Carbohydrate derivative binding	26	4.00×10^{-4}

BP, biological process; CC, cellular component; MF, molecular function; GO, gene ontology; cGMP, cyclic guanosine monophosphate.

expression levels of these 11 genes in the samples from obese and lean children are shown in Fig. 4A.

The transcription regulatory network of these marker genes is shown in Fig. 4B. The transcription factors with an NES score >5 were EP300 (E1A Binding Protein P300, NES=6.63), FOXA3 (Forkhead Box A3, NES=6.286), SRY (Sex Determining Region Y, NES=6.11), SNAI2 (NES=5.925), SRF (Serum Response Factor, NES=5.64), MAFA (MAF BZIP Transcription Factor A, NES=5.464), GCM1 (Glial Cells Missing Homolog 1, NES=5.157) and VDR (Vitamin D (1,25-Dihydroxyvitamin D3) Receptor, NES=5.115).

Discussion

In the present study, a total of 199 DEGs (79 up and 120 down-regulated genes) were identified in samples from obese children compared with those from lean children within the array dataset GSE29718. The results suggested that MMP9 and ACACB had

Table II. KEGG and Reactome pathway enrichment analyses for differentially expressed genes (P<0.05).

A, KEGG pathway

ID	Description	No. of genes	P-value
Upregulated			
04514	Cell adhesion molecules	4	1.12×10^{-2}
04145	Phagosome	4	1.80×10^{-2}
00520	Amino sugar and nucleotide sugar metabolism	2	4.01×10^{-2}
04670	Leukocyte transendothelial migration	3	4.11×10^{-2}
05144	Malaria	2	4.47×10^{-2}
05320	Autoimmune thyroid disease	2	4.63×10^{-2}
Downregulated			
00910	Nitrogen metabolism	3	1.00×10^{-3}
00640	Propanoate metabolism	3	2.60×10^{-3}
04270	Vascular smooth muscle contraction	4	1.77×10^{-2}
05412	Arrhythmogenic right ventricular cardiomyopathy	3	2.60×10^{-2}
00250	Alanine, aspartate and glutamate metabolism	2	3.11×10^{-2}
00564	Glycerophospholipid metabolism	3	3.18×10^{-2}
05414	Dilated cardiomyopathy	3	4.28×10^{-2}

B, Reactome pathway

ID	Description	No. of genes	P-value
Upregulated			
168256	Immune system	12	4.20×10^{-3}
1280218	Adaptive immune system	7	9.10×10^{-3}
1474244	Extracellular matrix organization	5	1.22×10^{-2}
2173782	Binding and uptake of ligands by scavenger receptors	2	2.10×10^{-2}
199992	Trans-golgi network vesicle budding	2	2.41×10^{-2}
421837	Clathrin-derived vesicle budding	2	2.41×10^{-2}
168249	Innate immune system	7	3.70×10^{-2}
1433557	Signaling by SCF-KIT	3	3.82×10^{-2}
2219530	Constitutive signaling by aberrant PI3K in cancer	2	4.32×10^{-2}
Downregulated			
5362517	Signaling by retinoic acid	4	4.00×10^{-4}
418457	cGMP effects	3	9.00×10^{-4}
392154	Nitric oxide stimulates guanylate cyclase	3	9.7×10^{-4}
109582	Hemostasis	10	3.00×10^{-3}
204174	Regulation of pyruvate dehydrogenase complex	2	4.10×10^{-3}

KEGG, Kyoto Encyclopedia of Genes and Genomes; cGMP, cyclic guanosine monophosphate; PI3K, phosphoinositide-3 kinase; SCF, stem cell factor.

a high degree of interaction in the PPI network and were marker genes in obese children. Moreover, upregulated DEGs were significantly enriched in Reactome pathways of the immune system, and in various associated terms of the GO-BP category with higher P-values. Furthermore, MMP9, an upregulated DEG, was enriched in the term immune system processes of the GO-BP category.

MMPs, a family of zinc-dependent endopeptidases, decrease components of basement membranes and extracellular matrix.

A previous study showed that the MMP9 is upregulated in obese children (32). Florys *et al* (33) reported increased levels of MMP9 in obese children with type 1 diabetes mellitus. Furthermore, MMP9 has a significant role in the expansion of fat cells and adipose tissue (34). It has been suggested that extracellular matrix remodeled by MMP9 regulated adipocyte differentiation, which then resulted in the progression of obesity (35). Moreover, The C^{-1562}T polymorphism of the MMP9 gene promoter may lead to the development of obesity (35).
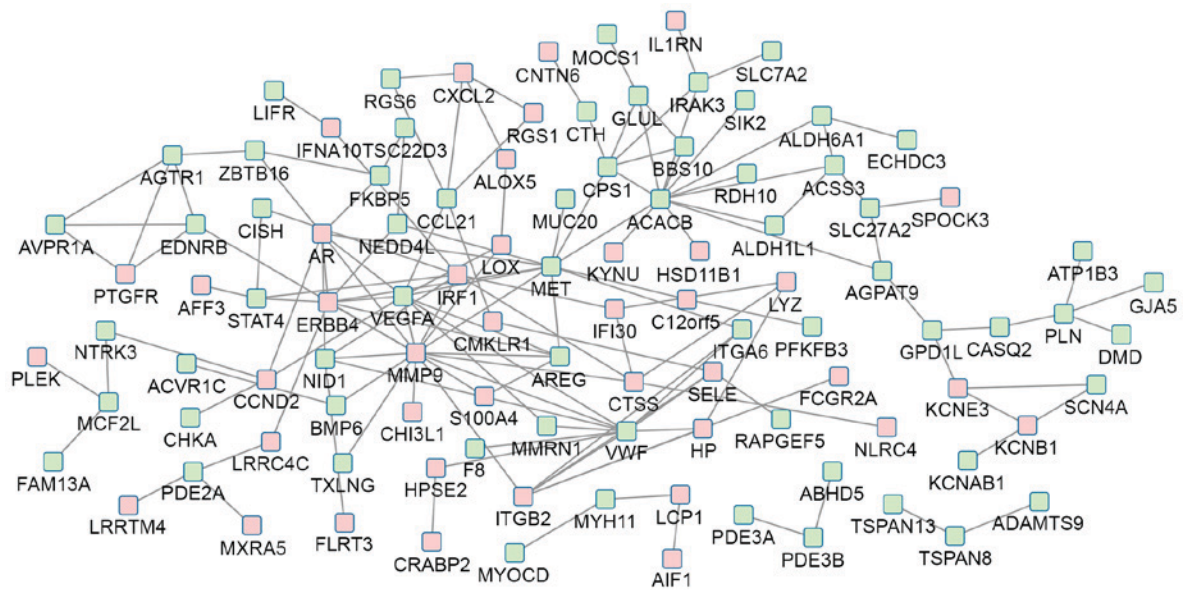
Figure 2. Protein-protein interaction network of differentially expressed genes. Red nodes represent upregulated and green nodes represent downregulated genes.
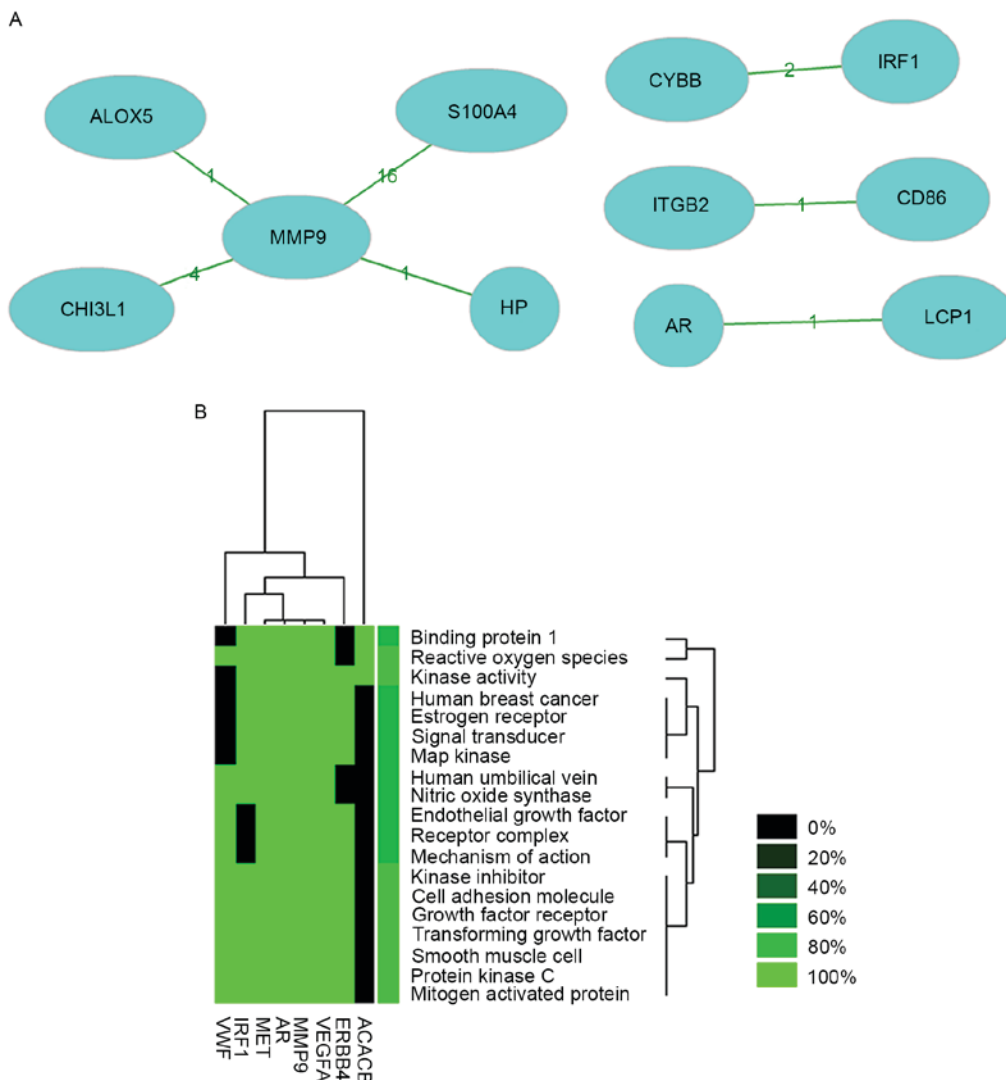

Figure 3. (A) Co-citation network of 8 genes with a higher degree of expression according to previous studies. The numbers on the linking lines indicate the number of studies co-cited. (B) Significant enrichment results of these 8 genes from previous studies. MMP, matrix metalloproteinase; ACACB, acetyl-CoA carboxylase β; VEGFA, vascular endothelial growth factor α; VWF, von Willebrand factor; AR, androgen receptor; IRF, interferon regulatory factor.
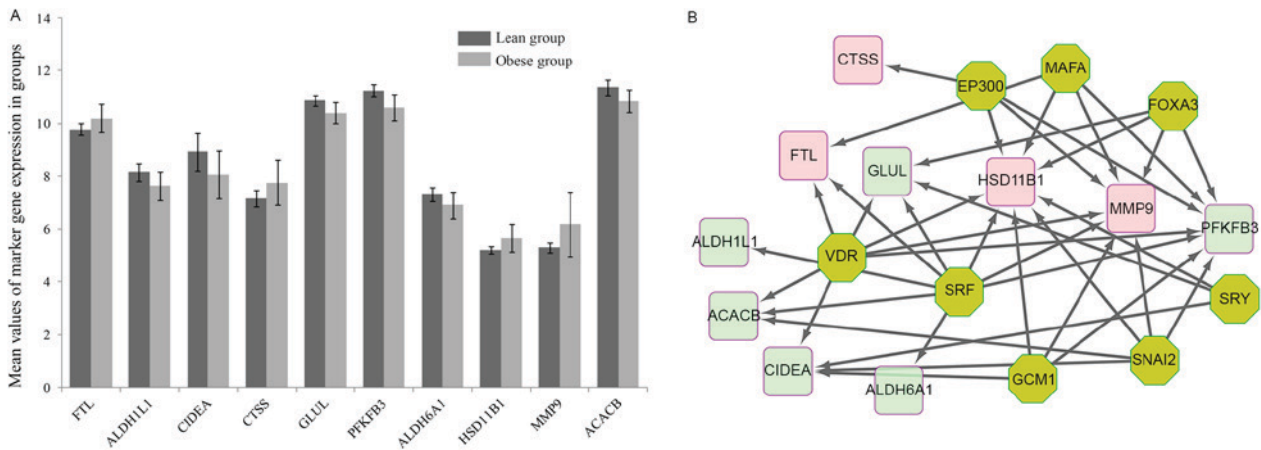
Figure 4. (A) Expression levels of 11 marker genes in samples from obese and lean children which had been listed as obesity-associated marker genes in the comparative toxicogenomics database. (B) Regulatory transcription factor network of the marker genes. MMP, matrix metalloproteinase; ACACB, acetyl-CoA carboxylase β. Red nodes represent upregulated genes, green nodes represent downregulated genes and yellow nodes represent regulating factors. EP300, E1A binding protein P300; FOXA3, forkhead box A3; SRY, sex-determining region; HSD11B1, hydroxysterol 11-β dehydrogenase 1; ALDH6A1, aldehyde dehydrogenase 6 family member A1; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase3; GLUL, glutamate-ammonia ligase; CTSS, cathepsin S; CIDEA, cell death-inducing DFFA-like effector A; FTL, ferritin light chain; VDR, vitamin D receptor; SRF, serum response factor; GCM1, glial cells missing homolog 1; SNAI2, Snail family transcriptional repressor 2.

Table III. Hub genes identified by the present study in the literature.

Gene	Co-genes (n)	Co-citations (n)	Total (n)
MMP9	4	22	18,527
ITGB2	1	1	6,736
ALOX5	1	1	325
CD86	1	1	7,281
S100A4	1	16	977
AR	1	1	13,054
HP	1	1	6,963
LCP1	1	1	289
CHI3L1	1	4	729
CYBB	1	2	2,048
IRF1	1	2	1,885

MMP, matrix metalloproteinase; AR, androgen receptor; IRF, interferon regulatory factor; ITGB2, integrin β2; ALOX5, arachidonate 5-lipoxygenase; S100A4, S100 calcium binding protein A4; HP, haptoglobin; LCP1, lymphocyte cytosolic protein 1; CHI3L1, chitinase 3-like 1; CYBB, cytochrome B-245 β chain. Co-genes indicate the number of linked genes. Co-citations indicate the number of other genes co-cited with this gene from literature mining.

In the present study, MMP9, which had the highest degree of interaction in the PPI network, was identified as a marker gene in obese children. Thus, the results of the present study are in accordance with those of previous ones and suggest that MMP9 may be a key gene associated with child obesity.

Furthermore, ACACB, which also had a high degree of interaction in the PPI network, was identified as a marker gene associated with child obesity in the present study. ACACB, one isoform of ACAC, is involved in fatty acid oxidation (36,37). The variants of the ACACB allele are associated with obesity (38). A previous study showed that body fat may act as a mechanism of metabolic adaptation and a signal that downregulated ACACB expression in adipose tissue (39). In addition, rs2268388 polymorphisms of ACACB are associated with severe obesity and ACACB has a significant part in disorders associated with energy metabolism such as obesity (38). Furthermore, ACACB is involved in triglyceride synthesis and triglyceride contents of soleus muscle were greater in obese than in lean individuals (40,41). Therefore, ACACB may be a key gene associated with obesity.

In addition, upregulated DEGs identified by the present study were found to be significantly enriched in Reactome pathways associated with the immune system. Obesity, malnutrition by excess, is associated with immune dysfunction (42). It has been suggested that the pathogenesis of obesity includes an impaired immune system and cell-mediated immune responses, and altered immune function (43). Besides, adipose tissues of obese individuals showed signs of immune response pathway activation compared with those in lean people in a study on weight-discordant twins (44). A previous study has demonstrated that the cell-mediated immune response is impaired in obese children (42). Tam *et al* (18) found an over-representation of the immune and inflammatory response in performing gene ontology analyses in adipose tissues of children. In addition, activation of JNK1 and inhibitor of κB kinase pathways regulate inflammatory and stress mechanisms in obesity, and numerous components of the immune system, such as macrophages, have roles in the pathologies of obesity-associated inflammation (45). The accumulation of macrophages and development of crown-like structures are involved in adipose tissue inflammation in obesity (45). Furthermore, obesity is associated with increased immune cells in the central nervous system (46). Therefore, pathways of the immune system may be significantly associated with child obesity. Besides, MMP9, an upregulated DEG, was enriched in the term immune system processes in the GO-BP category in

the present study. It is thus speculated that MMP9 may be involved in child obesity via immune system pathways.

The results of the present study showed that MMP9, ACACB and immune system pathways may have important roles in child obesity. Manco *et al* (47) suggested that massive weight loss had effects on the innate immune system in morbidly obese women. Laimer *et al* (48) indicated that weight loss in obese women was associated with decreased MMP9. Thus, MMP9 and immune system pathways may also have significant roles in adult obesity. However, to the best of our knowledge, no previous study found that ACACB was directly associated with the development of adult obesity, and thus, this gene may not have any direct relevance in adult obesity. ACACB may be differentially expressed in child obesity compared with adult obesity, which remains to be clarified in future studies. Genes and pathways associated with child and adult obesity as well as their differences are still required to be fully elucidated.

In conclusion, MMP9, ACACB and immune system pathways may have significant roles in child obesity. However, further studies on key genes and pathways associated with child obesity are required. In addition, the molecular mechanisms of adult obesity are required to be elucidated and to be compared with those of child obesity to identify differences between them.

Acknowledgements

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