Original Research

Identification of differentially expressed genes related to metabolic syndrome induced with high-fat diet in E3 rats

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Abstract

Understanding the genes differentially expressing in aberrant organs of metabolic syndrome (MetS) facilitates the uncovering of molecular mechanisms and the identification of novel therapeutic targets for the disease. This study aimed to identify differentially expressed genes related to MetS in livers of E3 rats with high-fat-diet-induced metabolic syndrome (HFD-MetS). E3 rats were fed with high-fat diet for 24 weeks to induce MetS. Then, suppression subtractive hybridization (SSH) technology was used to identify the genes differentially expressed between HFD-MetS and control E3 rat livers. Twenty positive recombinant clones were chosen randomly from forward subtractive library and sent to sequence. BLAST analysis in GenBank database was used to determine the property of each cDNA fragment. In total, 11 annotated genes, 3 ESTs, and 2 novel gene fragments were identified by SSH technology. The expression of four genes (*Alb*, *Pip4k2a*, *Scd1*, and *Tf*) known to be associated with MetS and other five genes (*Eif1*, *Rnase4*, *Rps12*, *Rup2*, and *Tmsb4*) unknown to be relevant to MetS was significantly up-regulated in the livers of HFD-MetS E3 rats compared with control rats using real-time quantitative PCR (RT-qPCR). By analyzing the correlations between the expression of these nine genes and serum concentrations of TG, Tch, HDL-C, and LDL-C, we found that there were significant positive correlations between TG and the expression of five genes (*Alb*, *Eif1*, *Pip4k2a*, *Rps12*, and *Tmsb4x*), Tch and three genes (*Rnase4*, *Scd1*, and *Tmsb4x*), and LDL-C and two genes (*Rnase4* and *Scd1*), as well there were significant negative correlations between HDL-C and the expression of three genes (*Rup2*, *Scd1*, and *Tf*). This study provides important clues for unraveling the molecular mechanisms of MetS.

Keywords: High-fat-diet-induced metabolic syndrome, suppression subtractive hybridization, differentially expressed genes

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Introduction

Metabolic syndrome (MetS) is a set of complex disorders characterized by central obesity, high blood pressure, hypercholesterolemia, hypertriglyceridemia, decreased high-density lipoprotein cholesterol, and glucose intolerance or type 2 diabetes.[1] Recent years have witnessed the rising morbidity of MetS with a trend to threaten younger population.[2] The etiopathogenesis of MetS is commonly considered as the complicated co-determination of genetic factors and environmental factors, but the detailed mechanisms behind the disease development are still under investigation.

Liver, as 'the metabolic center', acts in whole-body metabolic homeostasis. Hepatic dysfunction results in several metabolic diseases such as hyperlipidemia, hyperglycemia, and hypertension. Non-alcoholic fatty liver disease (NAFLD) is a common manifestation of MetS.[3,4] The prevalence of NAFLD increases by 70–90% in patients with obesity or diabetes,[5–7] and there are also great correlations between NAFLD and cardiovascular diseases.[8,9] So, the identification of differentially expressed genes in the liver of MetS will facilitate the understanding of molecular mechanisms for MetS development.

At present, methods such as representational difference analysis technology (RDA),[10–12] serial analysis of gene expression, cDNA microarray technology,[13,14] and subtractive hybridization technology (SSH), have been widely used to identify the genes differentially expressed in diseased tissues. cDNA microarray, with the advantages of speediness and possibility to assay thousands of genes, is commonly used to analyze known transcripts. RDA technology retains weakness, such as high false positive rates and annoying steps, and is not the efficient method to study the change of gene expression. The SSH technology

based on suppressed PCR and differential hybridization, can effectively enrich the genes differentially expressed between two kinds of tissues or cells, especially for low expressed ones.[15,16] The other superiority of SSH includes efficiency, easy operation, and a short experimental period. Currently, SSH technology has been broadly used and successfully identified differentially expressed genes in diverse pathological conditions.[17–21]

In this study, we used SSH technology to establish differentially expressed forward cDNA library between HFD-MetS E3 rat livers and control ones. As a result, we identified nine annotated genes that significantly up-regulated in HFD-MetS E3 rat livers and these gene expressions had significant relationships with certain serum parameters of MetS.

Materials and methods

Rats

E3 rats were bred in a specific pathogen-free animal house (SPF level). All procedures in this experiment were in accordance with institutional guidelines for animal research. The experiments were approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University.

Induction of MetS in E3 Rats

E3 rats were divided into HFD group and control group carefully matched for age and sex in the experiment. Each group contained five male and five female E3 rats aged 8–10 weeks, and the rats were fed with HFD and standard rat diet respectively for 24 weeks. The induction and evaluation of the rat HFD-MetS model were the same as previously described.[22] Briefly, during the experimental period, body weight was measured per week; glucose tolerance test (GTT) was conducted with Accu-CHEK Active (Roche, Basel, Switzerland) after 15 h fasting per 4 weeks, respectively at 0-, 30-, 60-, and 120-min time points after i.p. injection of D-glucose (2 g/kg body weight). At the end of 24-week HFD treatment, rats were sacrificed after being fasted for 15 h and the liver tissues and fasting blood samples were collected and stored at −85°C until use. The fasting serum concentrations of triglyceride (TG), total cholesterol (Tch), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured by the corresponding testing kits.

Total RNA isolation, mRNA purification, and cDNA synthesis

Total RNA from rat livers was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and mRNA was purified from total RNA with Poly A Tract® mRNA Isolation System (Promega, Madison, WI, USA). After detecting the mRNA quantity and quality, the first-strand cDNA with 2 μg mRNA of each sample as the template and the double-stranded cDNA were synthesized with the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

Construction of subtracted cDNA library

SSH was performed with the PCR-Select™ cDNA subtraction kit (Clontech, Palo Alto, CA, USA). Forward SSH (tester: the cDNA from MetS rat livers, driver: the cDNA from control rat livers) was carried out according to the manufacturer's protocol. Subtraction efficiency was evaluated using the subtracted and unsubtracted cDNAs as templates, by using *β-actin* as an internal standard. All the sequences of used adaptors and primers are depicted in Table 1.

The 2nd PCR products of SSH were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instruction and the recombinant plasmids were transformed into JM109 super strain cells.

Sequencing and real-time quantitative PCR

Twenty positive bacterial clones were randomly selected from forward subtractive cDNA library and sent to the company (BGI, BJ, China) for sequencing. Alignments were performed in the databases of GenBank against reference mRNA sequence (others) database, non-human, non-mouse ESTs (est_others) database, and genome database of rats.

For confirming the results from SSH, we selected four genes (*Alb, Pip4k2a, Scd1*, and *Tf*) that have been known to be associated with MetS and other five genes (*Eif1*, *Rnase4*, *Rps12*, *Rup2*, and *Tmsb4*) that are unknown to be relevant to MetS, and analyzed their expressions in HFD-MetS group

Table 1 Adaptor and primer sequences for SSH

Name	Sequence
adaptor 1	5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′ 3′-GGCCCGTCCA-5′
adaptor 2 R	5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3′ 3′-GCCGGCTCCA-5′
PCR primer 1	5′-CTAATACGACTCACTATAGGGC-3′
Nested PCR primer 1	5′-TCGAGCGGCCGCCCGGGCAGGT-3′
Nested PCR primer 2 R	5′-AGCGTGGTCGCGGCCGAGGT-3′
β-actin 1	5′-CACCCGCGAGTACAACCTTC-3′
β-actin 2 R	5′-CCCATACCCACCATCACACC-3′

and control group by using real-time quantitative PCR (RT-qPCR). Information regarding primers and products is depicted in Table 2.

Statistical analysis

Data were expressed as means ± SEM. The statistical analysis of differences between experimental groups was performed by Mann–Whitney *U*-test or Student's *t*-test. The correlations between the expression of nine annotated genes (*Alb*, *Eif1*, *Pip4k2a*, *Rnase4*, *Rps12*, *Rup2*, *Scd1*, *Tf*, and *Tmsb4x*) and the concentrations of serum TG, Tch, LDL-C, and HDL-C were performed by Pearson correlation ($n = 10$). $P < 0.05$ was considered significant.

Results

Evaluation of HFD-MetS in E3 rats

After the induction of MetS for 24 weeks, GTT in E3 rats in HFD group showed that the blood glucose was significantly higher than that of control group ones at 30-, 60-, and 120-min time points (Figure 1(a)). In addition, the concentrations of serum TG, Tch, and LDL-C were all significantly higher in E3 rats fed with HFD, compared with those in control group (Figure 1(b) to (d)). On the contrary, the concentration of serum HDL-C was significantly lower in HFD group than that in control group (Figure 1(e)). These data demonstrated that the E3 rats had succumbed to severe dyslipidemia and dysglycemia after being fed with HFD for 24 weeks.

Suppression subtractive hybridization

To identify the differentially expressed genes related to HFD-MetS in E3 rat livers, the forward SSH cDNA library containing 300 positive clones was constructed. The size of the 2nd PCR products from forward SSH library ranged from 100 bp to 1000 bp (Supplementary Figure A). The subtraction efficiency test indicated the successful subtraction in forward SSH (Supplementary Figure B). The colony PCR was used to identify the positive recombinant clones (Supplementary Figure C).

Table 2 Primer information for RT-qPCR

Gene	Sequence (5′–3′)	Size (bp)	T_M (°C)
Alb	P1: ATCCTCCTGCCTGCTACG	154	60
	P2: GGTGCTTTCTGGGTGTATCG		
Eif1	P1: ATCGTATCGTATGTCCGCTATC	139	60
	P2: GGTCTTCCTGCCGTTTCTC		
Pip4k2a	P1: GCAGCACCACCAGAGAAG	103	60
	P2: TCATCACCACCATCATCACC		
Rnase4	P1: CCTTGCTTCTGCTCTTGTTG	167	60
	P2: AGTCATCCTCCGTCTCTGC		
Rps12	P1: GCTGCTGGAGGTGTAATGG	116	60
	P2: CGCTTGTCTAAGGCTTTGG		
Rup2	P1: CCTATCTTGCTGCTTCCACTG	104	60
	P2: ACACGACAAAGTCCTGTAGAATC		
Scd1	P1: ATCTTCCTCATCATTGCCAACACC	189	60
	P2: TCCGCCCTTCTCTTTGACAGC		
Tf	P1: TGCTCTGCCTTGACAATACC	107	60
	P2: CTCTTTGCCATCTCCATTTCG		
Tmsb4x	P1: TTCCACGAGCATTGCCTTC	134	60
	P2: GCCTTCCTGGTCAGTAGTTC		

Sequencing of positive clones and RT-qPCR

Twenty clones were randomly selected from the forward subtractive library and sent to sequence. After BLAST analysis in GenBank database, 11 annotated genes (4 genes including *Eif1*, *Rps12*, *Scd1*, and *Tmsb4x* had 2 fragment clones), 3 ESTs, and 2 novel gene fragments were obtained (Table 3).

Nine annotated genes, including *Alb*, *Eif1*, *Pip4k2a*, *Rnase4*, *Rps12*, *Rup2*, *Scd1*, *Tf*, and *Tmsb4x*, were chosen for further study. In total, the mRNA expressions of nine target genes were all significantly up-regulated in HFD-MetS E3 rat livers, compared with control ones. Among these genes, four genes (*Alb*, 0.60 ± 0.12 in control group, 1.21 ± 0.15 in HFD-MetS group, $P < 0.01$; *Pip4k2a*, 0.80 ± 0.07 in control group, 1.55 ± 0.26 in HFD-MetS group, $P < 0.05$; *Scd1*, 0.10 ± 0.03 in control group, 0.42 ± 0.12 in HFD-MetS group, $P < 0.05$; *Tf*, 0.09 ± 0.03 in control group, 0.45 ± 0.16 in HFD-MetS group, $P < 0.05$) have been reported to be related with MetS (Figure 2(a)); the other five genes (*Eif1*, 0.78 ± 0.10 in control group, 1.30 ± 0.13 in HFD-MetS group, $P < 0.01$; *Rnase4*, 0.46 ± 0.07 in control group, 0.78 ± 0.13 in HFD-MetS group, $P < 0.05$; *Rps12*, 0.74 ± 0.07 in control group, 1.34 ± 0.21 in HFD-MetS group, $P < 0.05$; *Rup2*, 0.74 ± 0.08 in control group, 1.23 ± 0.15 in HFD-MetS group, $p < 0.05$; *Tmsb4x*, 0.42 ± 0.09 in control group, 1.19 ± 0.32 in HFD-MetS group, $p < 0.05$) have not been reported to be related with MetS thus far (Figure 2(b)).

Correlations between specific genes and metabolic phenotypes

The statistical analysis indicated that there existed several significant correlations between the relative expression of target genes and certain serum parameters of MetS. In details, there were five genes (*Alb*, *Eif1*, *Pip4k2a*, *Rps12*, and *Tmsb4x*) significantly correlated with serum TG concentration, three genes (*Rnase4*, *Scd1*, and *Tmsb4x*) significantly correlated with serum Tch, two genes (*Rnase4* and *Scd1*) significantly correlated with LDL-C and three genes (*Rup2*, *Scd1*, and *Tf*) significantly correlated with HDL-C (Table 4).

Discussion

In this study, we constructed the forward cDNA library of differentially expressed genes in HFD-MetS E3 rat livers, and identified 11 annotated genes, 3 rat ESTs, and 2 novel gene fragments as up-regulated genes associated with MetS. The expression of these annotated genes was significantly correlated with certain serum parameters of MetS, which will provide useful information for exploring the

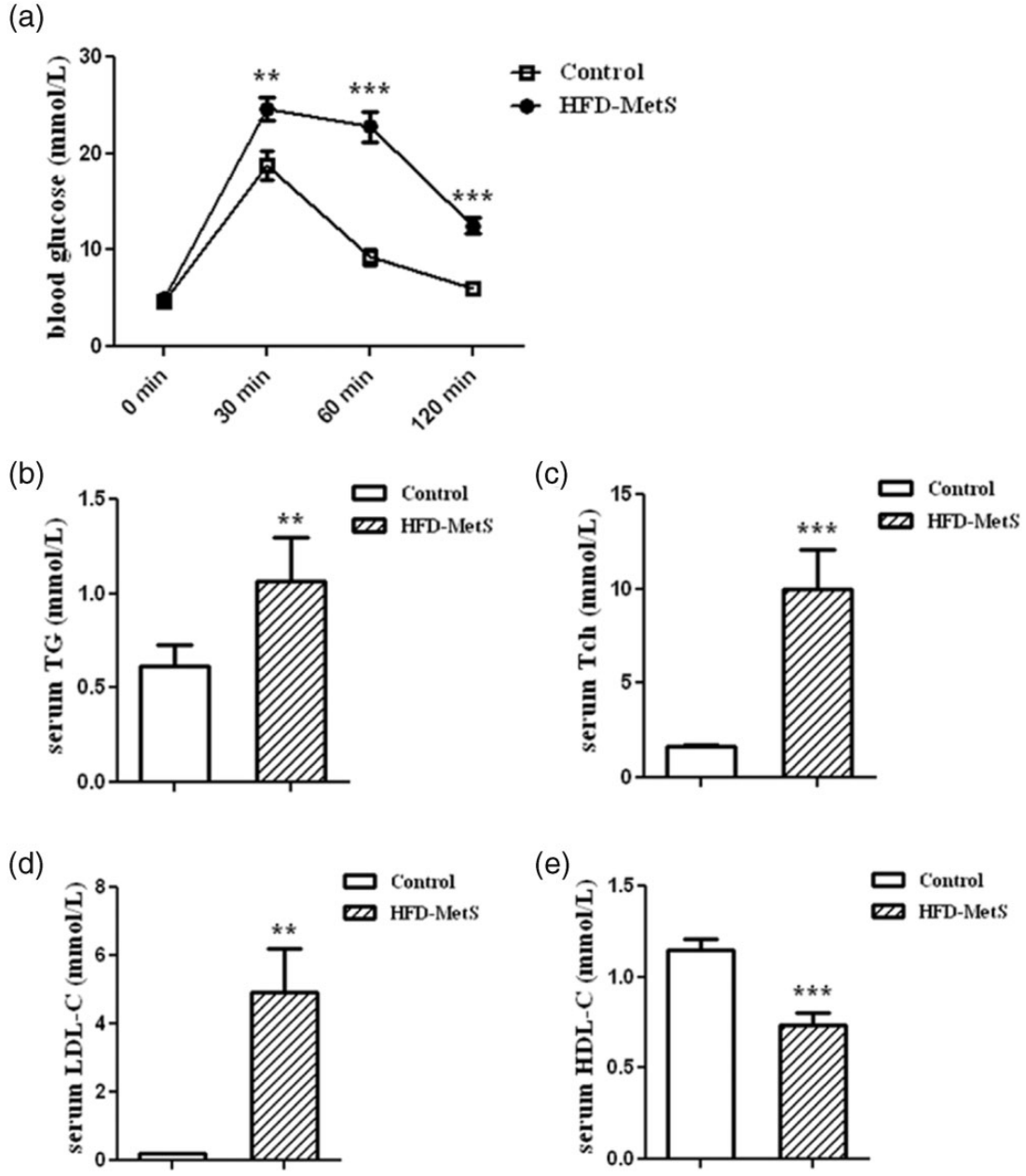

Figure 1 Evaluation of HFD-MetS E3 rat model after 24-week HFD treatment. (a) GTT was performed (0-, 30-, 60-, and 120-min time points) after the i.p. injection of D-glucose (2 g/kg body weight) by Roche glucose meter after 15 h fasting. (b) After fasted for 15 h, rats were sacrificed at the end of 24-week HFD treatment and the fasting blood samples were collected. The serum concentration of TG was detected by automatic biochemistry analyzer. (c) The serum concentration of Tch was detected. (d) The serum concentration of LDL-C was detected. (e) The serum concentration of HDL-C was detected. Data are expressed as means ± SEM. ** and *** represent $P < 0.01$ and $P < 0.001$, respectively, in the serum parameters between HFD-MetS group and control group ($n = 10$, each group had 5 male and 5 female rats). In HFD-MetS group the rats were fed with HFD, and in the control group the rats were fed with standard rat particle fodder.
GTT: glucose tolerance test; TG: triglyceride; Tch: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol

function of these genes in MetS pathogenesis. In addition, new ESTs and genes are under investigation.

There have been many studies focused on the differentially expressed genes related to MetS by the use of animal models. In the livers of HFD-mice, genes associated with adipogenesis and cholesterol synthesis are down-regulated, including *squalene epoxidase* (*Sqle*), *NAD(P)-dependent steroid dehydrogenase-like* (*Nsdhl*), *farnesyl diphosphate farnesyl transferase 1* (*Fdft1*), and *sterol-C4-methyl oxidase-like* (*Sc4mol*).[23] However, in epididymal fat pads of HFD-rats, *stearoyl-CoA desaturase* (*Scd*) is strongly up-regulated as 7-fold compared with normal rats.[24] Other studies showed that, in the livers of HFD-MetS-resistant mice, genes participating in the triglyceride synthesis are down-regulated,[25] and those controlling fatty acid β-oxidation, such as *acyl-CoA oxidase 1*(*Aco1*) and *HMG-CoA lyase*, are up-regulated.[23] In the rats with non-alcoholic steatohepatitis (NASH), the expressions of several genes related to lipid metabolism such as *perilipin* (*Plin*), *stearoyl-Coenzyme A desaturase 2* (*Scd2*), *ELOVL fatty acid elongase 3* (*Elovl3*) are increased; genes involved in

Table 3 BLAST alignment of differentially expressed genes in HFD-MetS liver of E3 rats

Official symbol	Full name	GenBank accession no.	Identity (%)	*E* value
Alb	Albumin	NM_134326.2	96	0.0
Eif1	Eukaryotic translation initiation factor 1	NM_001105837.1	100	1 e–158
Pip4k2a	Phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	NM_053926.2	99	2 e–165
Rnase4	Ribonuclease, Rnase A family 4	NM_020082.2	97	5.00 e–143
Rps12	Ribosomal protein S12	NM_031709.3	99	0.0
Rps24	Ribosomal protein S24	NM_031112.1	100	1 e–144
Rup2	Urinary protein 2	NM_001034950.2	99	8 e–135
Scd1	Stearoyl- Coenzyme A desaturase 1	NM_139192.2	99	6 e–134
Sftpc	Surfactant protein C	NM_017342.1	99	0.0
Tf	Transferrin	NM_001013110.1	100	5 e–121
Tmsb4x	Thymosin beta 4, X-linked	NM_031136.1	98	0.0

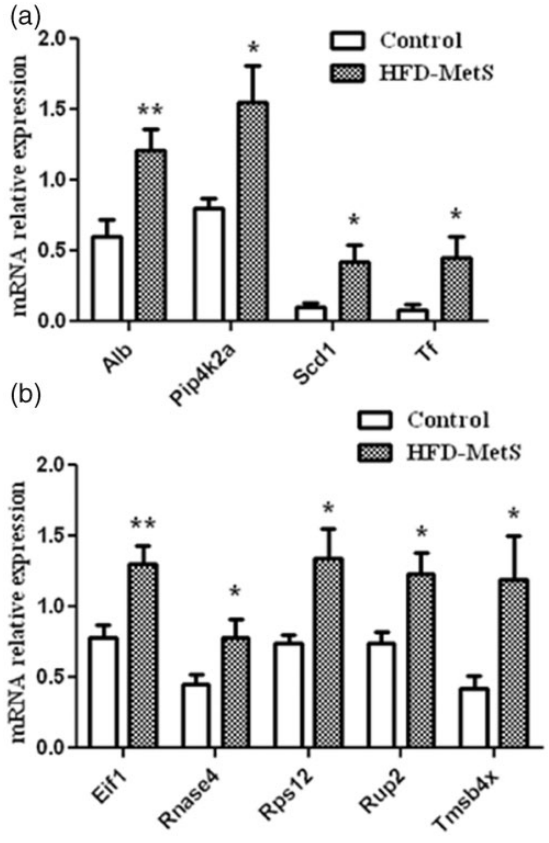

Figure 2 Identified gene expression determined by RT-qPCR. (a) The relative expression of 4 clarified genes was detected by RT-qPCR in livers from control group and HFD-MetS group. (b) The relative expression of 5 unclarified genes in livers from control group and HFD-MetS group. Data are expressed as means ± SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ between control group and HFD-MetS group ($n = 10$, each group had 5 male and 5 female rats). In HFD-MetS group the rats were fed with HFD, and in the control group the rats were fed with standard rat particle fodder.
Alb: *albumin*; *Pip4k2a*: *phosphate 4-kinase, type II, alpha*; *Scd1*: *stearoyl-coenzyme A desaturase 1*; *Tf*: *transferrin*; *Eif1*: *eukaryotic translation initiation factor 1*; *Rnase4*: *ribonuclease, Rnase A family 4*; *Rps12*: *ribosomal protein S12*; *Rup2*: *urinary protein 2*; *Tmsb4x*: *thymosin beta 4, X-linked*

cholesterol synthesis are down-regulated, including *3-hydroxy-3-methyl-glutaryl-Coenzyme A synthase 1* (*Hmgcs1*), *HMG-CoA reductase* (*Hmgcr*), *isopentenyl-diphosphate delta isomerase* (*Idi1*), *squalene epoxidase* (*Sqle*).[26] It also showed that the mRNA expression level of rate-limiting enzyme PFK1 is increased; and some others associated with carbohydrate metabolism are down-regulated, including *insulin-like growth factor binding protein 1* (*Igfbp1*, maintaining blood sugar level) and *one cut homeobox 1* (*Onecut1*, inducing the gene expression of *glucose kinase* and *glucose-6-phosphase*).[26] As inflammation is believed to go with metabolic disorders, there has many up-regulated inflammation-related genes in NASH rats, such as *Vnn1*, *Cxc19*, and *Bcl6*.[26] In diabetes mice, α1-serine protease inhibitors and some cytokines related to inflammatory response are up-regulated.[27]

As described in the studies above, our discovery provides valuable information on novel targets and useful methods for the mechanism research of MetS. Among the 11 annotated genes identified as up-regulated in HFD-MetS rat livers, 4 genes (*Alb*, *Pip4k2a*, *Scd1*, *Tf*) have been reported to be associated with MetS. *Alb*, located in human chromosome 4q13.3 and rat chromosome 14p22, has been defined as playing a role in nitric oxide signaling and the formation of S-nitrosothiols. The high level of serum albumin is associated with increased prevalence of MetS.[28] In addition, insulin can up-regulate the expression of *Alb* both on mRNA level and protein level.[29–31] Consistent with existing studies, we also found that the mRNA level of albumin was higher in HFD-MetS group compared with normal one, and revealed the significant correlation between *Alb* expression and serum TG concentration. *Pip4k2a*, located in human chromosome 10p12.2 and rat chromosome 17q12.3, has the ability to catalyze the synthesis of phosphatidylinositol 4, 5-bisphosphate. PIP4K2A takes part in the process of insulin resistance through determining the whole-body insulin responsiveness and specifically increases insulin sensitivity in skeletal muscle.[32,33] Up-regulated *Pip4k2a* induces the insulin resistance in HFD-MetS, and this would directly lead to the metabolic disturbance of glucose, lipid and protein.[34] All the disorders would raise many kinds of risk factors and cause diabetes or cardiovascular diseases finally.[34] *Scd1* maps to rat chromosome 1q54 and its homologous gene *Scd* maps to human chromosome 10q24.31. Its transcription product functions in the alteration from endogenous saturated fatty acids (SFA) to monounsaturated fatty acids (MUFA). SCD1 inhibition can

Table 4 Correlations between gene expressions and metabolic phenotypes (*r*)*

	TG	Tch	LDL-C	HDL-C
Alb	0.47*	0.19	0.18	−0.46
Eif1	0.66**	0.05	0.05	−0.36
Pip4k2a	0.68***	−0.09	−0.10	−0.33
Rnase4	−0.04	0.57**	0.56*	−0.39
Rps12	0.65**	−0.03	−0.03	−0.35
Rup2	0.81	−0.28	−0.09	−0.45*
Scd1	−0.21	0.57**	0.56*	−0.46*
Tf	−0.14	0.45*	0.41	−0.56*
Tmsb4x	0.78***	−0.06	−0.07	−0.47

The correlation was analyzed by using Pearson's correlation including all rats from HFD-MetS and control groups ($n = 20$).
*$P < 0.05$,
**$P < 0.01$,
***$P < 0.001$.

protect animals against HFD-induced obesity, hepatic steatosis, and insulin resistance.[35–37] *Scd1* deficiency causes the SFA accumulation in body and then leads to inflammation.[38] In conclusion, the increased activity of SCD1 induced by HFD-MetS will increase the TG concentration. The higher TG content leads to lipid metabolic disorders, and as a result, insulin resistance and MetS occur.[39] *Tf* maps in rat chromosome 8q32 and in human chromosome 3q22.1, and plays a role in iron transport and homeostasis.[40] There was a study showing that the higher incidence of MetS is accompanied by the higher content of transferrin and ferritin;[41] however, the regulation mechanism of *Tf* in the process of MetS is still waiting for clarification.

The seven other annotated genes (*Eif1*, *Rnase4*, *Rps12*, *Rps24*, *Rup2*, *Sftpc*, and *Tmsb4x*) identified in this study may become new targets for MetS research. eIF1, encoded by the *Eif1* gene, is critical for transfer of the initiator Met-tRNAf to form the pre-initiation complex.[42] Ribonuclease 4, as an enzyme encoded by the *Rnase4* gene, involves in mRNA cleavage.[43] RPS12 and RPS24 consist of the ribosomal subunits in conjunction with rRNA, and take part in the process of translation and DNA repair, and it has been reported that some ribosomal proteins are up-regulated in tumor tissues compared with normal ones.[44] Considering the information above, we inferred that the four genes may regulate the process of MetS by controlling the process of transcription and translation. In addition, *Tmsb4x* has been reported to take part in the inflammation and tissue repair,[45,46] which seems to be involved in inflammatory process of MetS.

In previous studies, the sequences of identified genes must be known, which limits the usage for discovering novel candidate genes. In addition, inbred E3 rat is susceptible to obesity and thus suitable for the study of MetS. Based on the two reasons, SSH, with its advantage that can easily find differential genes and whether the gene sequences are known or not, was used in our study to identify the differentially expressed genes in HFD-MetS E3 rat livers. As a result, several candidates genes significantly related to MetS have been obtained from our experiment. Further study will be focused on the research of their functions. The whole work will set up the foundation for elucidating molecular mechanism of MetS.

AUTHOR CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; XL and DL conducted the whole experiment, BZ provided necessary technical guides. JR, XW, and QS provided assistant work related to this experiment; YL, LL, and LL were in charge of the preparation work; SL was responsible for all the required funds raising, experiment designing, and paper appraisal as the study director. XL and DL contributed equally to this work.

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