

Genomic expression profiling and bioinformatics analysis on diabetic nephrology with ginsenoside Rg3

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Abstract. Diabetic nephropathy (DN), a common diabetes-related complication, is the leading cause of progressive chronic kidney disease (CKD) and end-stage renal disease. Despite the rapid development in the treatment of DN, currently available therapies used in early DN cannot prevent progressive CKD. The exact pathogenic mechanisms and the molecular events underlying DN development remain unclear. Ginsenoside Rg3 is a herbal medicine with numerous pharmacological effects. To gain a greater understanding of the molecular mechanism and signaling pathway underlying the effect of ginsenoside Rg3 in DN therapy, an RNA sequencing approach was performed to screen differential gene expression in a rat model of DN treated with ginsenoside Rg3. A combined bioinformatics analysis was then conducted to obtain insights into the underlying molecular mechanisms of the disease development, in order to identify potential novel targets for the treatment of DN. Six Sprague-Dawley male rats were randomly divided into 3 groups: Normal control group, DN group and ginsenoside-Rg3 treatment group, with two rats in each group. RNA sequencing was adopted for transcriptome profiling of cells from the renal cortex of DN rat model. Differentially expressed genes were screened out. Cluster analysis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were used to analyze the differentially expressed genes. In total, 78 differentially expressed genes in the DN control group were identified when compared with the normal control group, of which 52 genes were

upregulated and 26 genes were downregulated. Differential expression of 43 genes was observed in the ginsenoside-Rg3 treatment group when compared with the DN control group, consisting of 10 upregulated genes and 33 downregulated genes. Notably, 21 that were downregulated in the DN control group compared with the control were then shown to be upregulated in the ginsenoside-Rg3 treatment group compared with the DN control group. In addition, 7 upregulated genes in the DN control group compared with the control were then shown to be downregulated in the ginsenoside-Rg3 treatment group compared with the DN control group. Cluster analysis based on differentially expressed genes indicated that the transcriptomes are quite different among the samples. Distinct GO terms associated with these groups of genes were shown to be enriched. KEGG pathway analysis demonstrated that differentially expressed genes were predominantly involved in the fatty acid metabolism pathway and peroxisome proliferator-activated receptor (PPAR) signaling pathway. To the best of our knowledge, this study was the first to present whole genome expression profiling in DN with ginsenoside-Rg3 treatment by RNA-Seq. A set of differentially expressed genes and pathways were identified. These data provided an insight into understanding the molecular mechanisms underlying the effect of ginsenoside-Rg3 treatment of DN.

Introduction

Diabetic nephropathy (DN) is a chronic microvascular complication of type 1 and type 2 diabetes mellitus and is the leading cause of progressive chronic kidney disease and end-stage renal disease, which puts a serious burden on the patient's family and on society (1).

The exact pathogenic mechanisms and the molecular events of DN remain unclear. Evidence suggests that environmental and genetic factors are involved in the development of DN (2). A number of factors are known to be critical in the development of DN, such as insulin-like growth factor 1 (IGF1), transforming growth factor beta, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), interleukins (ILs), tumor necrosis factor- α (TNF- α), vascular endothelial growth factor (VEGF) and endothelin (3). These factors effect processes including glucose metabolism, renal hemodynamics,

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cell matrix metabolism, cell proliferation, cell hypertrophy, cell apoptosis, abnormal angiogenesis and cytokine-mediated inflammatory response, which are involved in DN pathogenesis. Searching for agents that regulate the expression of these cytokines to intervene and prevent the development of DN an area of research.

Ginsenoside Rg3 is an active ingredients in Ginseng, which can inhibit the growth and metastasis of tumors through the downregulation of VEGF, bFGF gene expression and inhibiting the formation of abnormal new blood vessels. Besides, ginsenoside Rg3 has a number of pharmacological effects, including the inducing the apoptosis of tumor cells, promoting T lymphocyte mitosis and NK cell activity, stimulating the phagocytic function of the reticuloendothelial system, promoting the secretion of B lymphocyte antibodies, promoting antiplatelet aggregation to prevent thrombosis, dilating the blood vessels and increasing blood supply (4,5).

Therefore, it was hypothesized that ginsenoside Rg3 could prevent the occurrence and development of DN by effecting the gene expression and regulation of various cytokines to delay the progression of DN to end-stage renal failure. To the best of our knowledge, this study was the first to observe the effect of ginsenoside Rg3 on the changes of the gene expression profile in DN rats, which may aid in the identification of novel targets for the treatment of DN.

Materials and methods

Animal group. Six male Sprague-Dawley (SD) rats were provided by Shanghai Laboratory Animal Center (Shanghai, China) (average weight, 300 g). The rats were divided randomly into three groups: Control group (sample ID: 1 and 3), DN control group (sample ID: 8 and 9) and ginsenoside-Rg3 treatment group (sample ID: 15 and 16), with two rats in each group.

Modeling and Rg3 drugs. The normal control group was given normal feed. The DN control and ginsenoside-Rg3 treatment groups were administered a high lipid diet for two months, then were treated with intraperitoneal injection of streptozotocin (STZ; 45 mg/kg) induced diabetic nephropathy. After the models were established, the ginsenoside-Rg3 treatment group were administered Rg3 (0.5 mg/kg) once a day for 30 days.

RNA extraction. Samples from renal cortex were collected and stored in liquid nitrogen at -80°C until RNA extraction was performed. Total RNA was extracted from the samples of renal cortex using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. RNA quantity and quality were measured using the NanoDrop ND-1000 spectrophotometer and Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA).

RNA-seq data generation. RNA-seq libraries were prepared in accordance with Illumina's sample preparation protocol. The libraries were sequenced onto an Illumina HiSeq2000 instrument (Illumina Inc., San Diego, CA, USA) and subjected to 100 cycles of paired end (2x100 bp) sequencing. The processing

Table I. Top ten enriched differentially expressed genes in the DN control group compared with the normal control group.

Gene	Description
Upregulated	
Angptl4	Angiopoietin-like 4
Hmgcs2	3-Hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
Havcr1	Hepatitis A virus cellular receptor 1
Gpx2	Glutathione peroxidase 2
Slc25a25	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25
Rarres2	Retinoic acid receptor responder (tazarotene induced) 2
Spp1	Secreted phosphoprotein 1
Serhl2	Serine hydrolase-like 2
Eci1	Enoyl-CoA Δ isomerase 1
Apoc2	Apolipoprotein C-II
Downregulated	
LOC361914	Similar to solute carrier family 7 (cationic amino acid transporter, y+system), member 12
Cacng5	Calcium channel, voltage-dependent, γ subunit 5
Slc7a12	Solute carrier family 7 (cationic amino acid transporter, y+system), member 12
LOC686288	Similar to olfactory receptor Olr1668
Prima1	Proline rich membrane anchor 1
Slco1a6	Solute carrier organic anion transporter family, member 1a6
RT1-CE1	RT1 class I, locus1
Hnmt	histamine N-methyltransferase
Cyp2c11	Cytochrome P450, subfamily 2, polypeptide 11
Cyp2c24	Cytochrome P450, family 2, subfamily c, polypeptide 24

of fluorescent images into sequences, base-calling and quality value calculations were performed using the Illumina data processing pipeline (version 1.8). Prior to assembly, high-quality clean reads were generated using FASTX toolkit pipeline (version 0.0.13), then the resulting high-quality reads were mapped onto the UCSC (mm10) using Tophat (version: 2.0.6) (6). Cufflink (version: 2.0.2) (7) was used to process the Tophat alignments. Additionally, transcript expression levels were estimated using Fragments Per Kilobases per Million reads (FPKM) values. Finally, the program Cuffdiff was used to define differentially expressed genes as a gene set for further analysis. The selection criteria of differentially expressed genes was based upon the fold-changes of the expression levels ($P < 0.05$). If the gene expression status was consistent in two comparison groups (DN control group vs. the normal control group including sample ID1 vs. ID8 and sample ID3 vs. ID9; ginsenoside-Rg3 treatment group vs. DN control group including sample ID8 vs. ID15 and sample ID9 vs. ID16), they were defined as differentially

Table II. Top ten enriched differentially expressed genes in the ginsenoside-Rg3 treatment group compared with the DN control group.

Gene	Description
Upregulated	
Cyp2c24	Cytochrome P450, family 2, subfamily c, polypeptide 24
Gtpbp4	GTP binding protein 4
Prima1	Proline rich membrane anchor 1
Slco1a6	Solute carrier organic anion transporter family, member 1a6
Prlr	Prolactin receptor
Tmem72	Transmembrane protein 72
Acadsb	Acyl-CoA dehydrogenase, short/branched chain
Ppm1k	Protein phosphatase, Mg ²⁺ /Mn ²⁺ dependent, 1K
LOC100365744	Hypothetical protein LOC100365744
Slc30a1	Solute carrier family 30 (zinc transporter, membrane 1)
Downregulated	
Havcr1	Hepatitis A virus cellular receptor 1
Ril	Reversion induced LIM gene
Hmgcs2	3-Hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
Slc25a25	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25
Cxcl11	Chemokine (C-X-C motif) ligand 11
Acaa1b	Acetyl-Coenzyme A acyltransferase 1B
Resp18	Regulated endocrine-specific protein 18
Rarres2	Retinoic acid receptor responder (tazarotene induced) 2
Rbp1	Retinol binding protein 1, cellular
Gdf15	Growth differentiation factor 15

expressed genes. All analyses were performed at Shanghai Biotechnology Corporation (Shanghai, China).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. In order to examine the biological significance of the differentially expressed genes, the GO and KEGG (<http://www.genome.jp/kegg/>) pathway analysis produced by Kanehisa Laboratories (Kyoto, Japan) were performed to investigate their functional and pathway annotation (8,9). GO and pathway analyses were conducted using TargetMine (available at <http://targetmine.nibio.go.jp/>) based on a hypergeometric test (10).

Results

Differentially expressed genes generated by RNA-Seq

Normal control group and DN control group. Totally, 78 differentially expressed genes in the DN control group were identified compared with the normal control group. Of

Table III. Twenty-eight differentially expressed genes.

Gene name	Description
LOC100365744	Hypothetical protein LOC100365744
Ppm1k	Protein phosphatase, Mg ²⁺ /Mn ²⁺ dependent, 1K
Acadsb	Acyl-CoA dehydrogenase, short/branched chain
Slco1a6	Solute carrier organic anion transporter family, member 1a6
Prlr	Prolactin receptor
Cyp2c24	Cytochrome P450, family 2, subfamily c, polypeptide 24
Prima1	Proline rich membrane anchor 1
Hmgcs2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
Eci1	Enoyl-CoA Δ isomerase 1
Gdf15	Growth differentiation factor 15
Plin2	Perilipin 2
Slc25a25	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25
Havcr1	Hepatitis A virus cellular receptor 1
Acaa1a	Acetyl-Coenzyme A acyltransferase 1A
Ephx1	Epoxide hydrolase 1, microsomal
Pck1	Phosphoenolpyruvate carboxykinase 1 (soluble)
Car15	Carbonic anhydrase 15
Apoc3	Apolipoprotein C-III
Ddit4l	DNA-damage-inducible transcript 4-like
Ril	Reversion induced LIM gene
Cyp4a1	Cytochrome P450, family 4, subfamily a, polypeptide 1
Rbp1	Retinol binding protein 1, cellular
Mt1a	Metallothionein 1a
Car4	Carbonic anhydrase 4
Gpx1	Glutathione peroxidase 1
Bhmt	Betaine-homocysteine S-methyltransferase
Rarres2	Retinoic acid receptor responder (tazarotene induced) 2
Resp18	Regulated endocrine-specific protein 18

these, 52 genes were upregulated while 26 genes were downregulated. The top ten enriched differentially expressed genes based on fold-change are listed in Table I.

DN control group and ginsenoside-Rg3 treatment group. In total, 43 differentially expressed genes in the ginsenoside-Rg3 treatment group were identified compared with the DN control group. Of these, 10 genes were upregulated while 33 genes were downregulated. The top ten enriched differentially expressed genes based on fold-change are listed in Table II.

Bioinformatics analysis. In total, 21 genes that were downregulated in the DN control group compared with the

Table IV. KEGG enrichment analysis of differential expressed genes.

Group	ID	Pathway	P-value	Count	Genes
Normal control vs. DN control group	rno03320	PPAR signaling pathway	4.09253x10 ⁻⁵	7	Acaa1a, Angptl4, Apoc3, Cyp4a1, Cyp4a3, Hmgcs2, Pck1
	rno04978	Mineral absorption	0.00179	5	Fth1, Mt1a, Mt2A, S100g, Tf
	rno00071	Fatty acid degradation	0.00199	5	Acaa1a, Acaa2, Cyp4a1, Cyp4a3, Eci1
DN control vs. ginsenoside-Rg3 treatment group	rno03320	PPAR signaling pathway	5.51233x10 ⁻⁵	6	Acaa1a, Acaa1b, Apoc3, Cyp4a1, Hmgcs2, Pck1
	rno00071	Fatty acid degradation	0.00908	4	Acaa1a, Acaa1b, Cyp4a1, Eci1

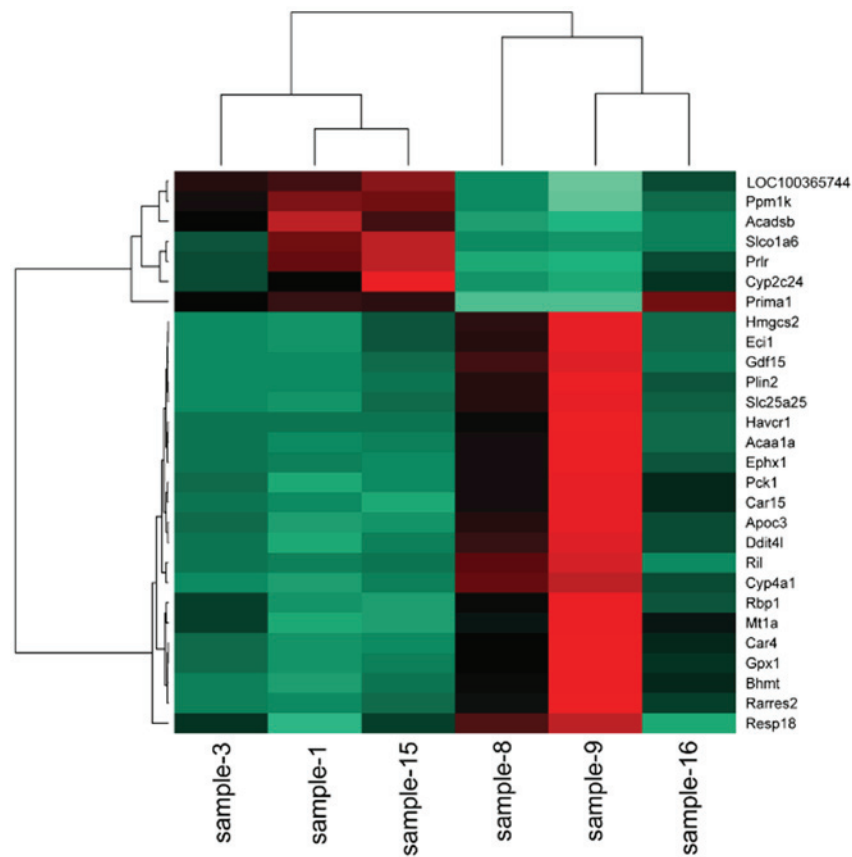


Figure 3. Hierarchical clustering of differentially expressed genes among six samples. Each row represents a differentially expressed gene, while each column represents a sample. In the heatmap, green represents genes that are downregulated red represents genes that are upregulated.

normal control group, were found to be upregulated in the ginsenoside-Rg3 treatment group compared with the DN control group. In addition, upregulation of 7 genes in the DN control group compared with the normal control group were downregulated in the ginsenoside-Rg3 treatment group compared with the DN control group. These 28 differentially expressed genes are shown in Table III.

Cluster analysis. To obtain a global view of the differentially expressed genes, the hierarchical cluster was performed among the samples. The heat maps indicated that the transcriptomes are different (Figs. 1-3).

GO and KEGG analysis. KEGG analysis was performed on the differentially expressed genes in the DN control group compared with the normal control group and in the

ginsenoside-Rg3 treatment group compared with the DN control group, respectively. Significantly affected pathways were identified in the upregulated gene sets in the normal control compared with the DN control group, and also in the downregulated gene sets in the DN control compared with the ginsenoside-Rg3 treatment group (Table IV).

The pathways, including peroxisome PPAR signaling pathway, mineral absorption and fatty acid degradation, may be critical in ginsenoside-Rg3 treatment of DN, implicating certain key genes enriched by these pathways (such as Acaala and Acaalb) as potential therapeutic targets.

To gain a greater understanding of the biological implications, GO enrichment analysis on differentially expressed genes was performed. GO is a standardized gene

Table V. GO analysis on 28 differentially expressed genes.

Ontology	GO ID	Term	P-value	Genes
GO_BP	GO:0006641	Triglyceride metabolic process	0.00194	GPX1, APOC3, PCK1
GO_BP	GO:0006639	Acylglycerol metabolic process	0.00242	GPX1, APOC3, PCK1
GO_BP	GO:0006638	Neutral lipid metabolic process	0.00260	GPX1, APOC3, PCK1
GO_BP	GO:0006720	Isoprenoid metabolic process	0.00260	RARRES2, HMGCS2, RBP1
GO_BP	GO:0006662	Glycerol ether metabolic process	0.00269	GPX1, APOC3, PCK1
GO_BP	GO:0018904	Organic ether metabolic process	0.00287	GPX1, APOC3, PCK1
GO_BP	GO:0009636	Response to toxin	0.00335	GPX1, MT1A, EPHX1
GO_BP	GO:0009725	Response to hormone stimulus	0.00380	GPX1, HMGCS2, APOC3, CAR4, PCK1
GO_BP	GO:0010033	Response to organic substance	0.00563	GPX1, HMGCS2, APOC3, EPHX1, CAR4, PCK1
GO_BP	GO:0009719	Response to endogenous stimulus	0.00576	GPX1, HMGCS2, APOC3, CAR4, PCK1
GO_BP	GO:0046486	Glycerolipid metabolic process	0.01784	GPX1, APOC3, PCK1
GO_MF	GO:0046914	Transition metal ion binding	0.02858	RIL, CYP4A1, MT1A, BHMT, CYP2C24, CAR4, PCK1
GO_BP	GO:0007584	Response to nutrient	0.02963	GPX1, HMGCS2, APOC3
GO_BP	GO:0043434	Response to peptide hormone stimulus	0.03400	HMGCS2, APOC3, PCK1
GO_MF	GO:0009055	Electron carrier activity	0.03467	ACADSB, CYP4A1, CYP2C24
GO_BP	GO:0009410	Response to xenobiotic stimulus	0.03516	GPX1, EPHX1
GO_BP	GO:0016101	Diterpenoid metabolic process	0.03899	RARRES2, RBP1
GO_BP	GO:0001523	Retinoid metabolic process	0.03899	RARRES2, RBP1
GO_BP	GO:0006721	Terpenoid metabolic process	0.04281	RARRES2, RBP1
GO_BP	GO:0055114	Oxidation reduction	0.04416	GPX1, ACADSB, CYP4A1, CYP2C24

GO_BP, biological process GO_MF, molecular function.

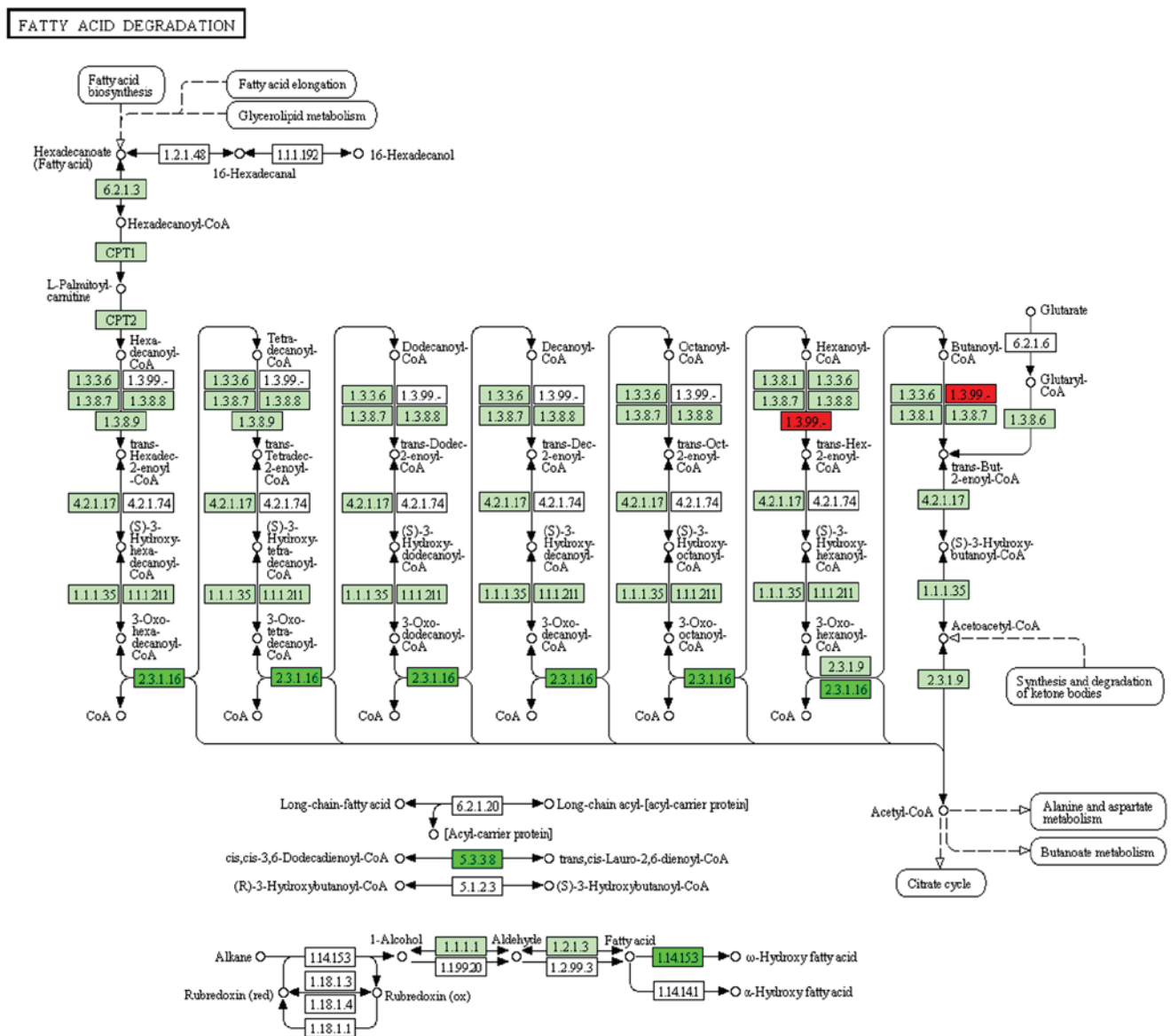
Table VI. KEGG enrichment analysis on 28 differentially expressed genes.

Pathway	Term	P-value	Count	Genes
rno03320	PPAR signaling pathway	7.88382×10^{-8}	5	Acaa1a, Apoc3, Cyp4a1, Hmgcs2, Pck1
rno00071	Fatty acid degradation	4.42130×10^{-5}	3	Acaa1a, Cyp4a1, Eci1
rno00910	Nitrogen metabolism	0.00029	2	Car15, Car4
rno04964	Proximal tubule bicarbonate reclamation	0.00049	2	Car4, Pck1
rno00280	Valine, leucine and isoleucine degradation	0.00286	2	Acaa1a, Hmgcs2

functional classification system, which provides a strictly defined concept and controlled vocabulary to describe properties of genes and their products. GO-annotated differentially expressed genes predominantly belong to the three functional clusters (biological process, cellular component and molecular function). Enrichment analysis focused on the 28 differentially expressed genes (21 upregulated genes and 7 downregulated genes) following administration of ginsenoside-Rg3. It was demonstrated that the 28 differentially expressed genes in the cluster of biological process was predominantly associated with lipid metabolism process (Table V). In addition, KEGG enrichment analysis was also performed on the 28 genes. Five pathways, including the

PPAR signaling pathway, fatty acid degradation, nitrogen metabolism, proximal tubule bicarbonate reclamation and valine, leucine and isoleucine degradation were shown to be enriched (Table VI).

Pathway analysis. Differentially expressed genes were mapped to rno00071 and rno03320 pathways of KEGG to observe the gene distribution and effects. In the rno00071 pathway, it was demonstrated that ginsenoside-Rg3 treatment reversed the expression of the acyl-CoA dehydrogenase, short/branched chain gene, acetyl-CoA acyltransferase, enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase and cytochrome P450 family 4 subfamily a polypeptide 2, suggesting a critical role of fatty acid metabolism pathway



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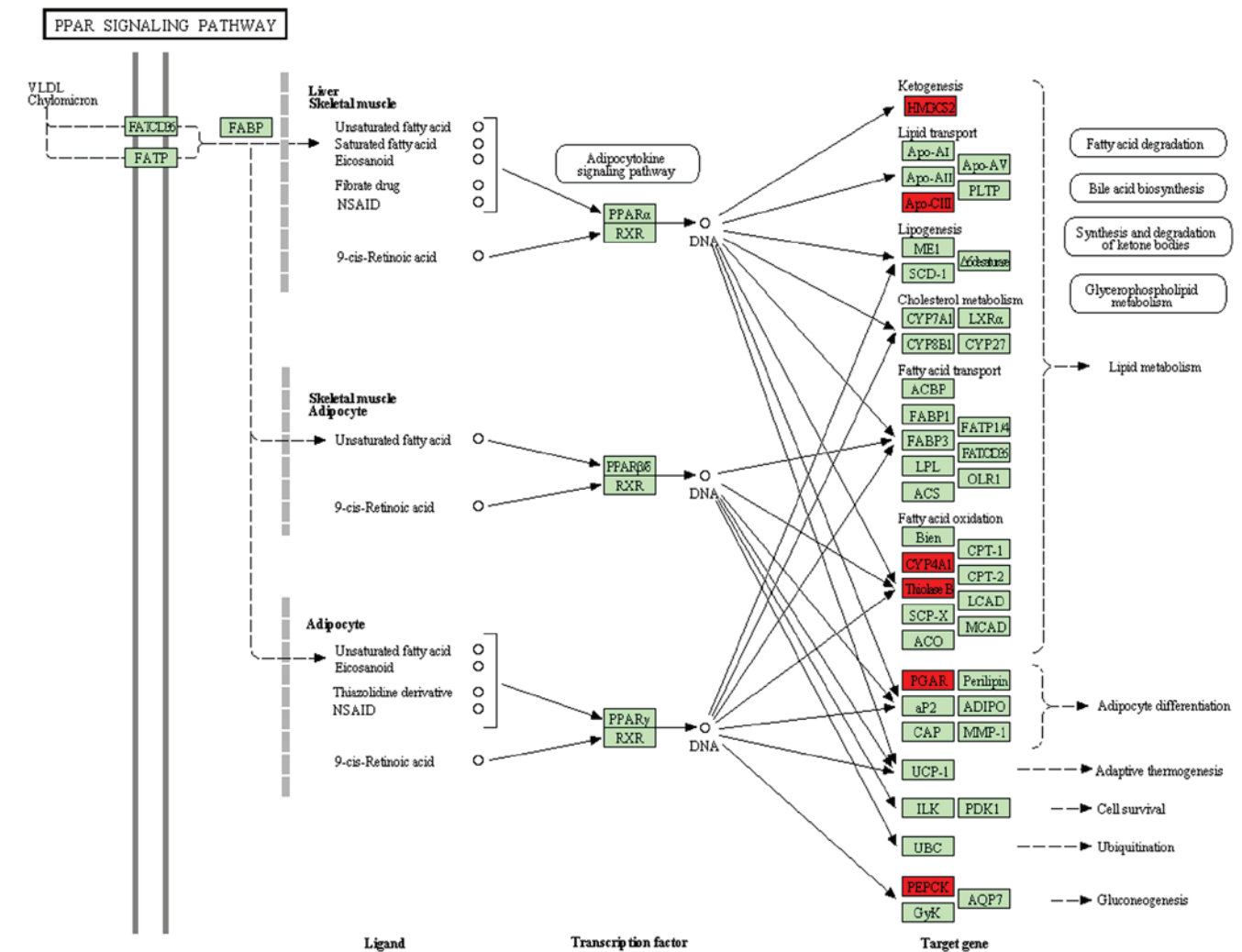
Figure 5. Differentially expressed gene distribution in the rno00071 pathway. Differentially expressed genes in the ginsenoside-Rg3 treatment group compared with the DN control group were shown to be associated with fatty acid degradation. Bright green, upregulated genes; red, downregulated genes; pale green, species specific genes.

of 10 upregulated and 33 genes downregulated. Notably, 21 downregulated genes in the DN control group were upregulated in the ginsenoside-Rg3 treatment group, and 7 upregulated genes in the DN control group were downregulated in the ginsenoside-Rg3 treatment group. GO annotation analysis showed that the differentially expressed genes were predominantly associated with the lipid metabolism process. KEGG pathway enrichment analysis showed that fatty acid degradation and PPAR signaling pathways were associated with the differentially expressed genes.

Recently, lipid metabolism disorder has become a focus of research in the pathogenesis of DN. Disordered lipid metabolism and renal lipid accumulation are not only associated with obesity-related renal disease and DN, but they may also contribute to the disease process (14). Sustained hyperglycemia in diabetes promotes fatty acid (FA) synthesis

and triacylglycerol (TG) accumulation. Elevated serum TG, free FAs (FFAs), and modified cholesterol cause ectopic lipid accumulation in nonadipose tissues, leading to lipotoxicity (15), which may be involved in the pathogenesis of DN (16). Herman-Edelstein *et al* (17) investigated the association of altered renal TG and cholesterol metabolism with lipid accumulation in patients with DN. The results showed a highly significant correlation between glomerular filtration rate, inflammation and lipid metabolism associated genes, suggesting a potential role of abnormal lipid metabolism in the pathogenesis of DN (17).

PPAR is a member of the nuclear hormone receptor superfamily and is critical in lipid metabolism (18). PPAR is highly expressed in various organs, such as the liver, renal cortex and heart. Knocking out PPAR α appeared to aggravate the severity of DN through an increase in extracellular matrix



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Figure 6. Differentially expressed gene distribution in the m03320 pathway. Differentially expressed genes in the DN control group compared with the normal control group were shown to be associated with the PPAR signaling pathway. Red, downregulated genes; pale green, species specific genes. PPAR, peroxisome proliferator-activated receptor.

formation, inflammation, and circulating FFA and TG concentrations (19). PPAR-γ is the most extensively studied PPAR subtype and is involved in adipocyte differentiation, and glucose and lipid metabolism. The mechanisms of PPAR-γ in DN remain to be fully elucidated. A study suggested PPAR-γ has an important role in regulating insulin sensitivity (20). Gene polymorphisms of PPAR-γ gene polymorphism Ala12 carriers exhibited an improvement in insulin sensitivity (21), and may be responsible for the development of DN.

Current therapeutic strategies for DN remain suboptimal and are directed at delaying disease progression, for example, intensive glucose and blood pressure control, dyslipidemia and lipid-lowering drugs. Ginsenoside Rg3, an active component of *Panax ginseng*, has been identified to have a protective effect against hyperglycemia, obesity and diabetes *in vivo* (22). Animal experiments demonstrated the effect of ginsenoside Rg3 on diabetic renal damage (23). However, the precise mechanisms of these actions remain to be fully elucidated. Hwang *et al* (24) investigated the molecular basis of ginsenoside Rg3. Their results found that the effect of ginsenoside Rg3 in inhibiting adipocyte differentiation, and also PPAR-γ signaling

was involved in the inhibition of adipocyte differentiation by ginsenoside Rg3 (24). Ginsenoside has been shown to exhibit anti-obesity and anti-hyperglycemia effects that involve the PPAR mediated pathway (25). Sun *et al* (26) investigated the effect of ginsenoside Rg3 on the expression of VEGF and TNF-α in the retina of diabetic rats, and demonstrated that ginsenoside Rg3 could downregulate the expression of VEGF and TNF-α, which may disrupt the development of diabetic retinopathy. Kang *et al* (22) analyzed the effect of ginsenoside Rg3 on the progression of renal disease in type II diabetic rat models, and provided evidence that Rg3 can prevent the progression of renal damage and dysfunction of diabetic rats. Lee *et al* (27) evaluated the effects of ginsenoside Rg3 on glucose uptake and the glucose transport system in mature 3T3-L1 cells, and demonstrated that ginsenoside Rg3 may stimulate the expression of insulin receptor substrate expression and phosphatidylinositol 3-kinase-110a protein, which may therefore be a valuable antidiabetic and antihyperglycemic agent. Bu *et al* (15) also demonstrated similar effects of ginsenoside Rg3 in reducing the fasting blood glucose level, reducing food and water intake, improving oral glucose tolerance, and repairing injured pancreas

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