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Gene expression analysis: Regulation of key genes associated with mycophenolate mofetil treatment of symptomatic carotid artery stenosis

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Abstract. The present study analyzed gene expression arrays to identify differentially-expressed genes (DEGs) between mycophenolate mofetil (MMF)-treated and placebo-treated patients with symptomatic carotid artery stenosis (SCAS). In addition, the key genes involved in the pharmacology of MMF treatment in patients with SCAS were identified. The gene expression dataset was obtained from a Gene Expression Omnibus database, which included 9 MMF-treated and 11 placebo-treated samples. The DEGs were identified between MMF and placebo groups using R software. Furthermore, a protein-protein interaction (PPI) network of the identified DEGS was constructed. The Database for Annotation, Visualization and Integrated Discovery was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the 19 most significant DEGs. A total of 210 DEGs between the MMF and placebo groups were screened and their PPI was constructed. GO function analysis revealed that the 19 DEGs were predominantly involved in the tyrosine phosphorylation of signal transducer and activator of transcription-5 protein, which is closely associated with the activation of T cells. The KEGG pathway analysis suggested that the main metabolic pathways of the 19 DEGs were associated with the pharmacological functioning of MMF in activated T cells. In conclusion, the present study identified numerous key DEGs associated with SCAS, and the results suggested that v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog and apelin may serve important roles in the MMF treatment of SCAS.

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Introduction

Stroke is the fourth leading cause of mortality and disability in the United States (1), and 10-20% of strokes are caused by carotid artery disease (2,3). According to epidemiological data, ~7 million adults in the United States have suffered a stroke (4), of which ischemic strokes account for ~90% (5). In addition, ~10% of ischemic strokes are caused by carotid artery stenosis (CAS) (6). CAS refers to a narrowing or constriction of the lumen of the carotid artery, usually attributed to atherosclerosis (7). The dynamic and complex process of atherosclerosis remains to be fully understood; however, it is well known that atherosclerosis is characterized by the accumulation of lipid particles and fibrous elements, associated with migration and proliferation of smooth muscle cells, in the large arteries (8-10).

Over the past decade, inflammation and the immune response in atherosclerosis have garnered attention. Previous studies have indicated that low-density lipoprotein particles and their content inside the vessel wall are susceptible to oxidation by free radicals, which may initiate the accumulation and invasion of macrophages, eventually lead to a narrowing of the major arteries (9,11).

During the progression of atherosclerosis, an imbalance between anti-inflammatory and proinflammatory cytokines serves an important role. Previous studies have attempted to identify the immune-associated genes that are involved in atherosclerosis, and achievements have been made (12,13). Superoxide dismutase, which is expressed at higher levels in regions of laminar flow, may combat oxidative stress, and hence limit vascular cell adhesion molecule-1 (VCAM-1) expression and the expression of other inflammatory pathways (12). Nitric oxide arises from endothelial nitric oxide synthase, which is known to be a shear stress-regulated gene, and can inhibit VCAM gene expression through a novel pathway involving inhibition of the activation of nuclear factor-κB,

the central transcription factor in vascular inflammation (13). In addition, previous studies have reported that interleukin (IL)-35 may upregulate the expression of anti-inflammatory cytokines (14-16). Huang *et al* (17) investigated the effects of IL-35 on atherosclerosis and hypothesized that IL-35 could be considered a novel target for the treatment of atherosclerosis. However, the majority of genes relevant to atherosclerosis remain unknown.

Mycophenolate mofetil (MMF) is an inhibitor of the enzyme inosine monophosphate dehydroxygenase (IMPDH), and exerts a powerful cytostatic effect on activated T cells by interfering with their DNA synthesis (18). In the present study, gene expression data were obtained from a Gene Expression Omnibus (GEO) dataset uploaded by van Leuven *et al* (19), which included 20 carotid endarterectomy samples from patients with CAS (>70% diameter stenosis on angiography) that were randomly assigned to the following treatment groups: Treatment with 1,000 mg MMF (n=9) or placebo (n=11). Patients were treated with MMF or placebo for ≥2 weeks prior to undergoing carotid endarterectomy (CEA). van Leuven *et al* (19) reported that the inflammatory process in human atherosclerotic plaques could be modified by short-term treatment with MMF, as determined using mRNA expression profiling. However, this previous study did not analyze the expression data in detail, nor did it determine how MMF functioned in the treatment of symptomatic CAS (SCAS) or the molecular mechanisms of SCAS.

In the present study, the gene expression data were used to identify differentially-expressed genes (DEGs) between MMF-treated and placebo-treated groups, with the aim of identifying potential genes associated with atherosclerosis, which may be considered targets for novel gene therapy. A total of 210 DEGs between the MMF and placebo groups were identified with a threshold of P<0.05. After analyzing the regulatory effects, a regulatory network was constructed based on the DEGs. Subsequently, the data were processed by bioinformatic analyses, including hierarchical clustering, Gene Ontology (GO) terms (molecular function, biological processes and cellular components) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Finally, the 19 most significant DEGs were screened; the results of these analyses indicated that apelin (APLN) and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) may be valuable for characterizing the mechanism underlying immunomodulatory therapy in atherosclerosis.

Materials and methods

Datasets. The GSE13922 original mRNA expression profile used in the present study was downloaded from the National Center of Biotechnology Information GEO (www.ncbi.nlm.nih.gov/geo/). The platform used to analyze these data was the GPL6255 Illumina humanRef-8 v2.0 expression beadchip (Illumina, San Diego, CA, USA).

Identification of DEGs. Background correction and quartile data normalization of the downloaded data were performed using the robust multi-array average (RMA) algorithm (20). Probes without a corresponding gene symbol were filtered and the average value of gene symbols with numerous probes was calculated. The expression profile dataset, including 13,985 genes for the 20 samples, was subsequently obtained. Student's t-test was used to identify DEGs between the MMF and placebo groups using the R software LIMMA package (version 3.3.1; www.r-project.org) (21). Genes with P<0.05 were considered DEGs and genes with P<0.01 were considered the most significant DEGs between the two treatment groups. The most significant DEGs were screened between the MMF and placebo groups using principal components analysis (PCA). Cluster analysis of the most significant DEGs was applied to generate a heat map, which allowed for visualization of the differential gene expression between the two groups.

Protein-protein interaction (PPI) network construction and analysis. The PPI network was constructed from 210 DEGs using the STRING online database (www.string-db.org/). PPI pairs with an interaction score >0.4 were used to construct the PPI network. Subsequently, the regulatory relationships between genes were analyzed according to the topological properties of the network. With a threshold of P<0.05 and |logFC|≥0.5, the key genes in the network were further screened.

Functional analysis of various DEGs. In order to identify biological functions associated with the pathogenesis of atherosclerosis, bioinformatics analyses, including hierarchical clustering, GO (22) terms (molecular function, biological processes, cellular components) analysis and KEGG (23) pathway analysis, were conducted for the most significant DEGs, using the online Database for Annotation, Visualization and Integrated Discovery tool (24), based on the method of Expression Analysis Systemic Explorer (EASE) test (25). The enrichment threshold was an EASE score of 0.1.

Results

Data processing. A total of 13,985 genes in 20 samples were obtained after preprocessing of the expression profile. The original expression datasets were processed into expression estimates using the RMA method. As presented in the box plot in Fig. 1, the median of different samples was almost the same following normalization, which indicated a great degree of standardization.

Identification of DEGs. The DEGs between the MMF and placebo groups were identified using LIMMA package (21) in R software. A total of 210 DEGs were identified with the threshold of P<0.05, including 19 most significant DEGs with the threshold of P<0.01 (Table I). Analysis of the most significant DEGs revealed that there were 14 up- and 5 downregulated genes (Fig. 2). As presented in Fig. 3, all of the selected DEGs were screened using PCA to distinguish between the MMF and placebo groups. In the first principal components, 50.82% of variances were explained, whereas in the second principal component, 9.73% of variances were explained. In total, the resolution degree of variances was 60.55%.

PPI network construction and analysis. A total of 189 PPI pairs were obtained from the STRING database. After wiping out

Table I. List of the 19 most significant DEGs (P<0.01).

Gene symbol	Gene name	P-value	logFC
AK4	Adenylate kinase 4	0.0042	0.4736
APLN	Apelin	0.0092	0.5261
CKB	Creatine kinase, brain	0.0011	0.9570
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	0.0097	0.2979
EBI3	Epstein-Barr virus induced 3	0.0048	-0.7416
ECE1	Endothelin converting enzyme 1	0.0088	0.1873
FAM102A	Family with sequence similarity 102, member A	0.0057	-0.2496
GFRA2	GDNF family receptor α2	0.0068	0.7860
GPM6B	Glycoprotein M6B	0.0064	0.7289
HDC	Histidine decarboxylase	0.0045	0.9700
KIT	V-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	0.0018	0.6283
LMO4	LIM domain only 4	0.0037	0.3623
MGST2	Microsomal glutathione S-transferase 2	0.0049	-0.3100
MRPL30	Mitochondrial ribosomal protein L30	0.0031	0.1626
NSDHL	NAD(P) dependent steroid dehydrogenase-like	0.0082	0.3269
POLR2I	Polymerase (RNA) II (DNA directed) polypeptide I, 14.5kDa	0.0053	0.2292
RRM1	Ribonucleotide reductase catalytic subunit M1	0.0094	0.2653
SCIN	Scinderin	0.0022	-0.9358
WDR41	WD repeat domain 41	0.0042	0.4693

FC, fold change.

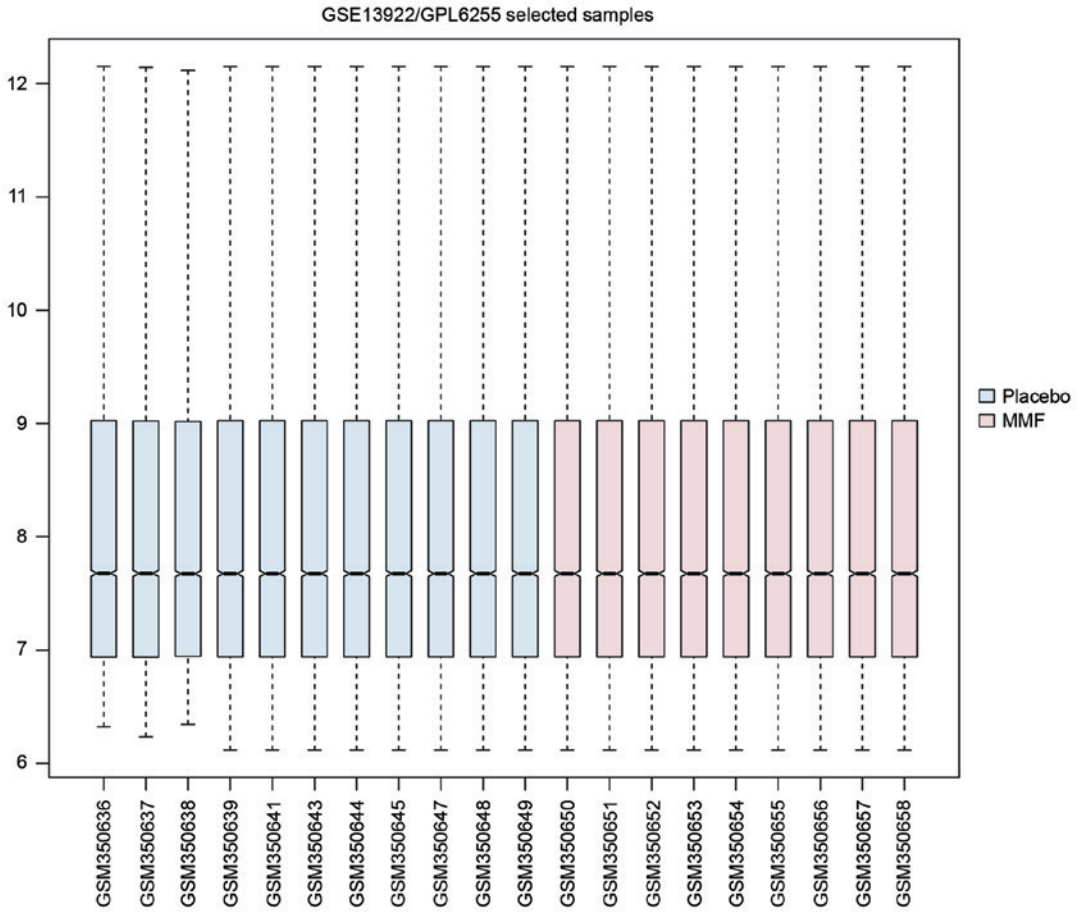

Figure 1. Box plot of the mRNA microarray datasets after normalization. The horizontal axis represents the name of the samples, whereas the vertical axis represents the expression value. Short black lines are used to identify the degree of standardization. MMF, mycophenolate mofetil.

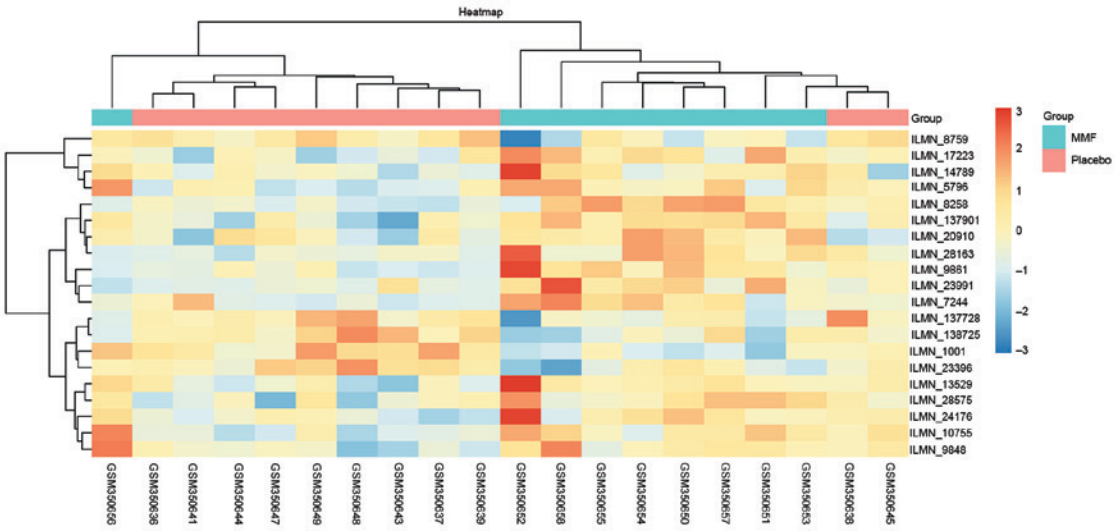

Figure 2. Hierarchical clustering dendrogram of gene expression. A total of 19 DEGs were screened in carotid endarterectomy samples from 11 MMF-treated and 9 placebo-treated patients with symptomatic coronary artery stenosis. A change in color from red to blue indicated high to low expression. DEGs, differentially-expressed genes; MMF, mycophenolate mofetil.

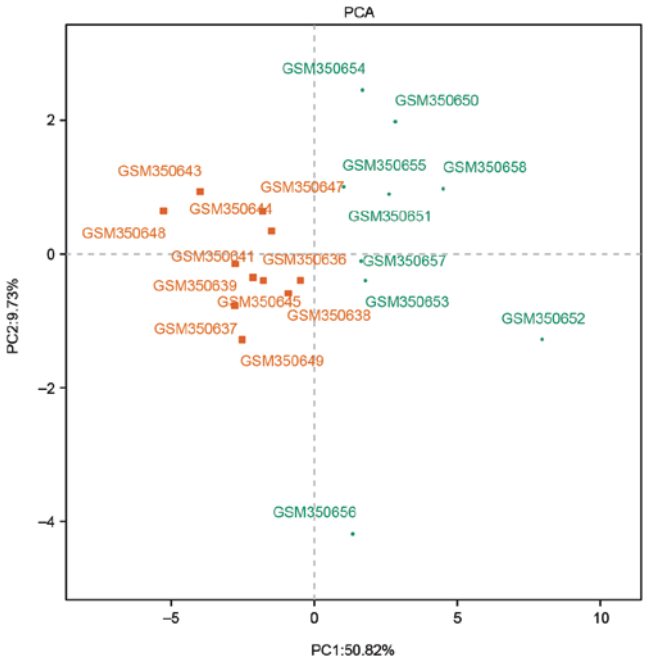

Figure 3. PCA of the 19 differentially-expressed genes from 20 samples. The horizontal axis represents the scores of first principal components and the vertical axis represents the scores of second principal components of samples. In the first principal components, 50.82% of variances were explained, whereas in the second principal component, 9.73% of variances were explained. Totally, the resolution degree of variances was 60.55%. PCA, principal components analysis.

the pairs isolated from the major network, the PPI network composed of 172 edges and 105 nodes (Fig. 4). In the network, nodes represent genes and edges represent the interaction between genes. The PPI network of DEGs was in a state of high aggregation, which is an essential property of biological networks. With a threshold degree ≥3, a total of 43 genes were selected (Table II). As shown in Fig. 4, tumor protein p53 (degree, 33) and E1A binding protein P300 (degree, 18) had more degrees than the other DEGs.

GO functional analysis. Using a threshold of P<0.05, a total of 401 significant GO terms were enriched and the top 10 enriched terms for each category are presented in Fig. 5; only 8 terms were enriched in cellular components. The most enriched GO terms of the DEGs were mainly associated with tyrosine phosphorylation of signal transducer and activator of transcription-5 (Stat5) protein, which is closely associated with the activation of T cells.

KEGG pathway enrichment analysis. Using a threshold of P<0.05, a total of 6 significant KEGG pathways were enriched (Fig. 6). The most enriched KEGG pathways of the DEGs were mainly associated with the pharmacological functioning of MMF in activated T cells, including purine metabolism, glutathione metabolism and pyrimidine metabolism.

Discussion

The treatment of CAS includes three modalities: Medical management, carotid artery angioplasty and stenting, and CEA (26). However, there are doubts regarding the efficacy of carotid artery angioplasty and stenting, and CEA, in specific patients (27-29). Previous studies have aimed to identify novel treatments and medications for atherosclerosis. Following construction of the hypercholesterolaemic apolipoprotein E-deficient murine model, Chen *et al* (30) demonstrated that preimplantation factor could prevent atherosclerosis via its immunomodulatory effects without affecting serum lipids. In addition, Sun *et al* (31) developed trifunctional Simian virus 40 (SV40)-based nanoparticles for *in vivo* targeting and imaging of atherosclerotic plaques, and targeted SV40 virus-like nanoparticles were revealed to deliver a greater concentration of the anticoagulant drug Hirulog to atherosclerotic plaques.

It is well known that MMF is a T cell suppressor, which is able to reduce synthesis of guanine via IMPDH inhibition, resulting in the suppression of T cell proliferation (32). van Leuven *et al* (19) demonstrated that treatment with

Table II. DEGs with degree ≥3.

Gene symbol	Gene name	P-value	logFC	Degree
CKB	Creatine kinase B	0.0011	0.957	3
KIT	KIT proto-oncogene receptor tyrosine kinase	0.0018	0.6283	10
AK4	Adenylate kinase 4	0.0042	0.4736	7
POLR2I	RNA polymerase II subunit I	0.0053	0.2292	4
APLN	Apelin	0.0092	0.5261	3
RRM1	Ribonucleotide reductase catalytic subunit M1	0.0094	0.2653	7
CSF2	Colony stimulating factor 2	0.0097	0.2979	7
ENO2	Enolase 2	0.0143	0.5576	4
NUP98	Nucleoporin 98	0.0174	0.1405	5
TLR4	Toll like receptor 4	0.0179	-0.422	8
NES	Nestin	0.0189	0.481	3
CD2BP2	CD2 cytoplasmic tail binding protein 2	0.019	0.1659	3
PPP3CA	Protein phosphatase 3 catalytic subunit α	0.019	0.2495	5
COPS6	COP9 signalosome subunit 6	0.0196	0.2328	4
FST	Follistatin	0.0199	0.7444	3
EP300	E1A binding protein p300	0.0202	0.2351	18
BBS10	Bardet-Biedl syndrome 10	0.0211	0.2222	5
MAP1B	Microtubule associated protein 1B	0.0215	0.1588	3
HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3	0.022	0.2015	5
PDE4D	Phosphodiesterase 4D	0.023	0.2095	4
SOD2	Superoxide dismutase 2, mitochondrial	0.0236	0.3115	5
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide	0.0252	0.3726	6
TFDP2	Transcription factor Dp-2	0.026	0.232	5
GPX1	Glutathione peroxidase 1	0.0261	-0.7343	4
PTGES	Prostaglandin E synthase	0.0275	0.2743	3
ACO1	Aconitase 1	0.0303	0.3555	7
RAN	RAN, member RAS oncogene family	0.0306	0.3282	3
COL5A3	Collagen type V α3 chain	0.0316	0.1173	3
USF1	Upstream transcription factor 1	0.0332	-0.4322	3
SOX9	SRY-box 9	0.0345	0.2414	7
PDE2A	Phosphodiesterase 2A	0.0356	0.1566	3
CHMP2A	Charged multivesicular body protein 2A	0.0361	0.1974	3
AK8	Adenylate kinase 8	0.0362	0.2411	8
DHX9	DEAH-box helicase 9	0.0367	0.1559	5
LEP	Leptin	0.0373	0.7399	7
TP53	Tumor protein p53	0.038	0.1518	33
YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase Activation protein epsilon	0.0383	0.1716	5
MCM4	Minichromosome maintenance complex component 4	0.0393	0.2495	5
PFKP	Phosphofructokinase, platelet	0.0395	0.3782	4
LMNA	Lamin A/C	0.0446	0.4168	5
CCT3	Chaperonin containing TCP1 subunit 3	0.0446	0.2518	5
BMP2	Bone morphogenetic protein 2	0.0458	0.1583	11
DYRK1A	Dual specificity tyrosine phosphorylation Regulated kinase 1A	0.0495	-0.2047	5

FC, fold change. Gene symbols shown in bold represent genes with P<0.01 and underlined genes represent the gene with |log fold change|≥0.5.

MMF was able to reduce inflammatory cell infiltration, with a concomitant decrease in proinflammatory gene expression in patients with SCAS. The present study downloaded and analyzed a GEO mRNA expression profile uploaded by van Leuven *et al* (19). A total of 210 DEGs were identified between MMF-treated and placebo-treated groups.

Table III. Most significantly enriched GO terms (P<0.001).

GO ID	GO term	Associated genes
GO:0048513	Organ development	AK4, APLN, CKB, CSF2, ECE1, GPM6B, KIT, LMO4, NSDHL, SCIN
GO:0048731	System development	AK4, APLN, CKB, CSF2, ECE1, GFRA2, GPM6B, KIT, LMO4, NSDHL, SCIN
GO:0042523	Positive regulation of tyrosine phosphorylation of Stat5 protein	CSF2, KIT
GO:0042522	Regulation of tyrosine phosphorylation of Stat5 protein	CSF2, KIT
GO:0042506	Tyrosine phosphorylation of Stat5 protein	CSF2, KIT
GO:0030219	Megakaryocyte differentiation	KIT, SCIN
GO:0048856	Anatomical structure development	AK4, APLN, CKB, CSF2, ECE1, GFRA2, GPM6B, KIT, LMO4, NSDHL, SCIN
GO:0007275	Multicellular organismal development	AK4, APLN, CKB, CSF2, ECE1, GFRA2, GPM6B, KIT, LMO4, NSDHL, SCIN
GO:0044087	Regulation of cellular component biogenesis	CSF2, GPM6B, LMO4, SCIN
GO:0034311	Diol metabolic process	AK4, HDC

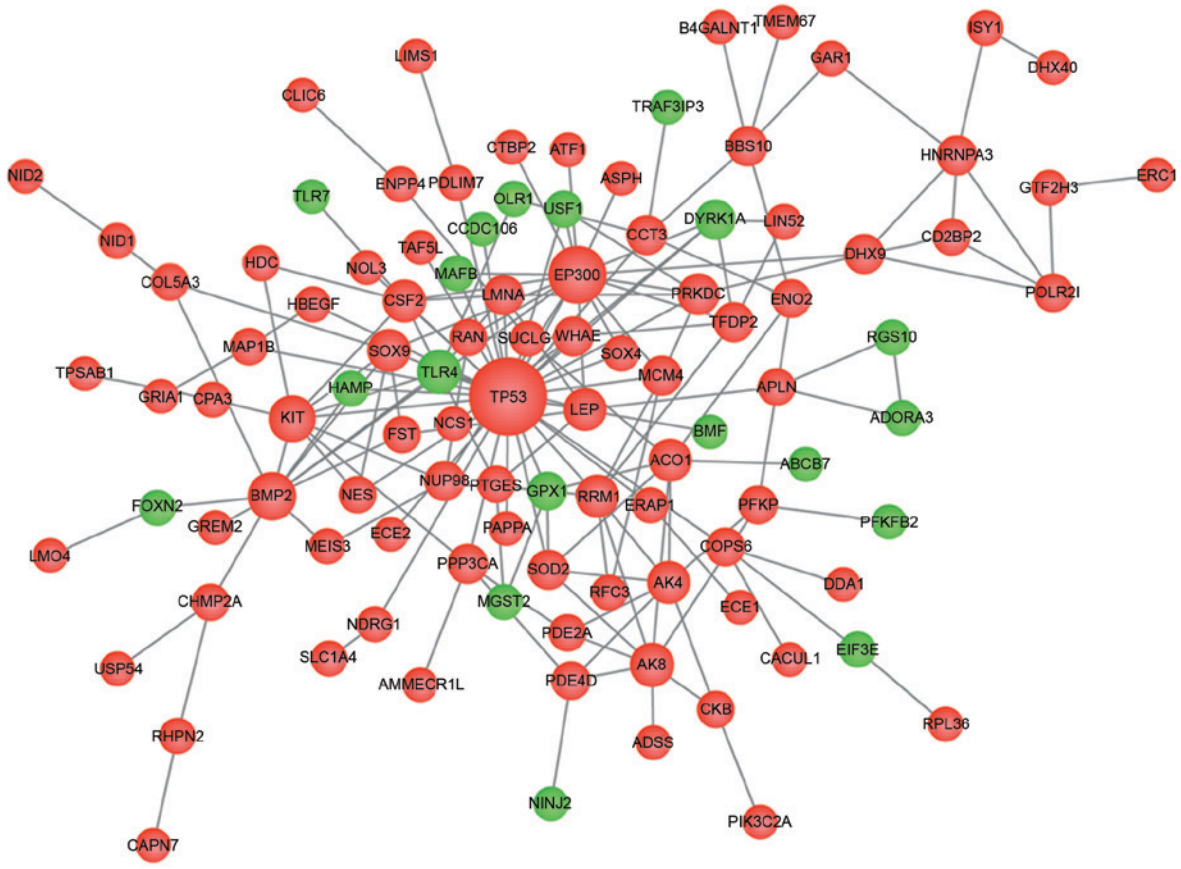

Figure 4. Protein-protein interaction network of DEGs. Red nodes represent upregulated DEGs, and green nodes represent downregulated DEGs; the connecting lines represent the interactions between DEGs. The size of the node is proportional to the degree of the DEG. DEGs, differentially-expressed genes.

Subsequently, the 19 most significant DEGs were selected to undergo GO functional analysis and KEGG pathway enrichment analysis. The results revealed that the most enriched KEGG pathway was purine metabolism, indicating that suppression of inflammatory activity served an important role in the MMF treatment of patients with SCAS. Furthermore,

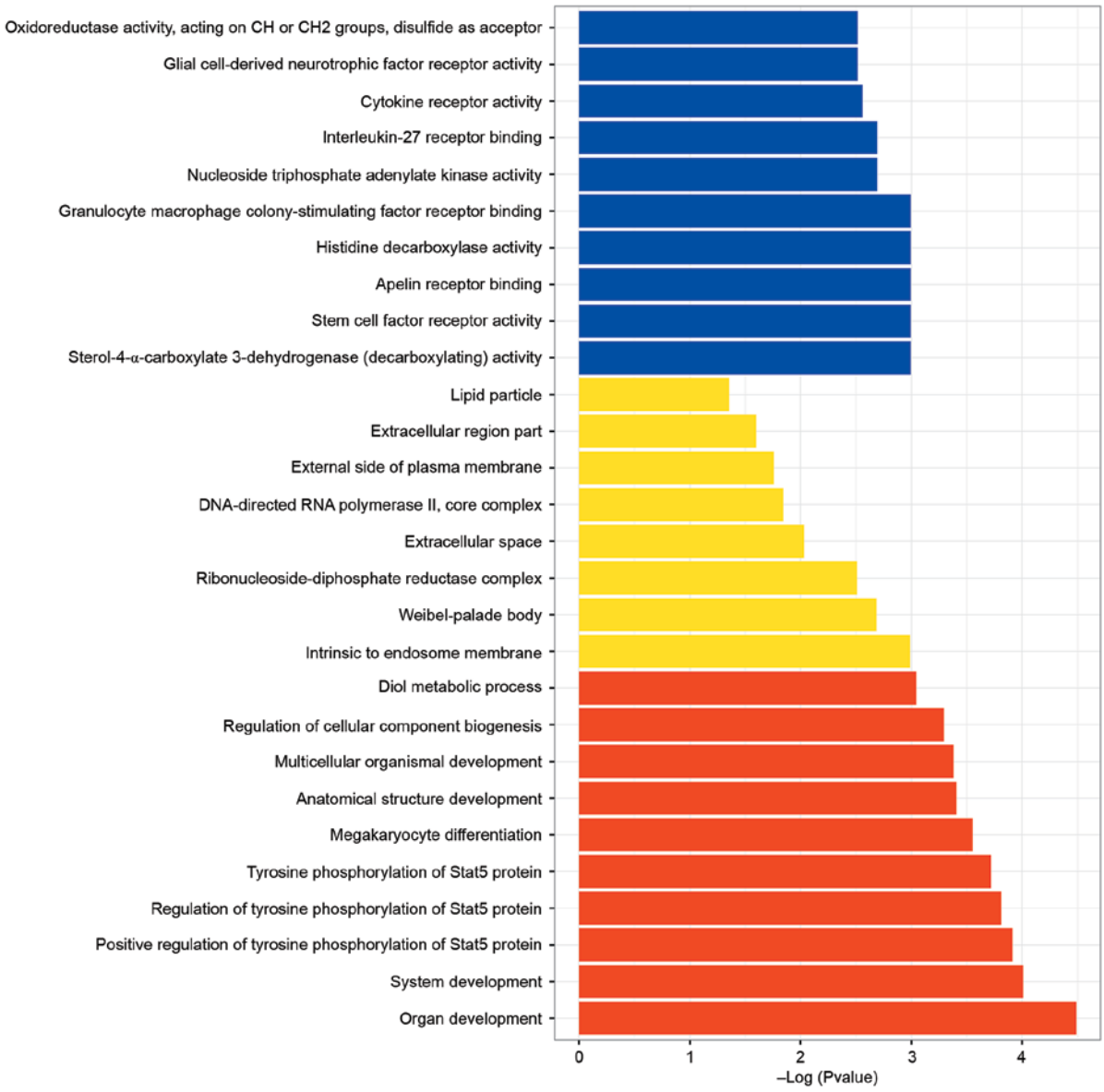

Figure 5. GO term analysis, separated into biological process, cellular component and molecular function categories, for differentially-expressed genes. Top 10 enriched terms of each category are shown; only 8 terms are presented for cellular components. Red bars represent biological process terms; yellow bars represent cell component terms; blue bars represent molecular function terms. The vertical axis represents the GO category and the horizontal axis represents the -log (p-value). P<0.05 was used as the threshold for selecting significant GO categories. GO, Gene Ontology.

with a threshold of degree ≥3 and |logFC|≥0.5, three genes, APLN, creatine kinase B (CKB) and KIT, were selected as the key genes.

APLN is a peptide, which was initially identified by Tatemoto *et al* in 1998 (33), that functions as an endogenous ligand for the orphaned G-protein-coupled receptor (APJ) (34). Previous studies have revealed that APJ deficiency can prevent oxidative stress-associated atherosclerosis and that the APLN-APJ system is a mediator of oxidative stress in vascular tissue (33,35-37). In the present study, expression of APLN was significantly different between MMF-treated and placebo-treated groups, and APLN was a key gene in the PPI network, indicating that APLN may be a target of MMF for the treatment of SCAS. According to the results of the KEGG analysis, purine metabolism was the most enriched signaling pathway, indicating that MMF modified the atherosclerotic plaque by suppressing activated T cells. Recent studies have also reported that APLN is involved in the immune response (38,39). By analyzing the expression and function of the APLN-APJ system in tumor vasculature, Kidoya *et al* (38) indicated that the APLN-APJ system could induce maturation of tumor vasculature and improve the efficiency of immune therapy. The results of the GO analysis suggested that terms associated with Stat5 were the most enriched, including tyrosine phosphorylation of Stat5 protein, regulation of tyrosine phosphorylation of Stat5 protein and positive regulation of tyrosine phosphorylation of Stat5 protein (Table III). Previous studies have indicated that Stat5 has a strong association with T cells. Lindahl *et al* (40) provided evidence to suggest that microRNA-21 is expressed *in situ* in cutaneous T cell lymphomas skin lesions, as induced by IL-2 and IL-15 cytokines, and is regulated by Stat5 in malignant T cells. The APLN-APJ system is also involved in the immune response and Stat3. Han *et al* (41) indicated

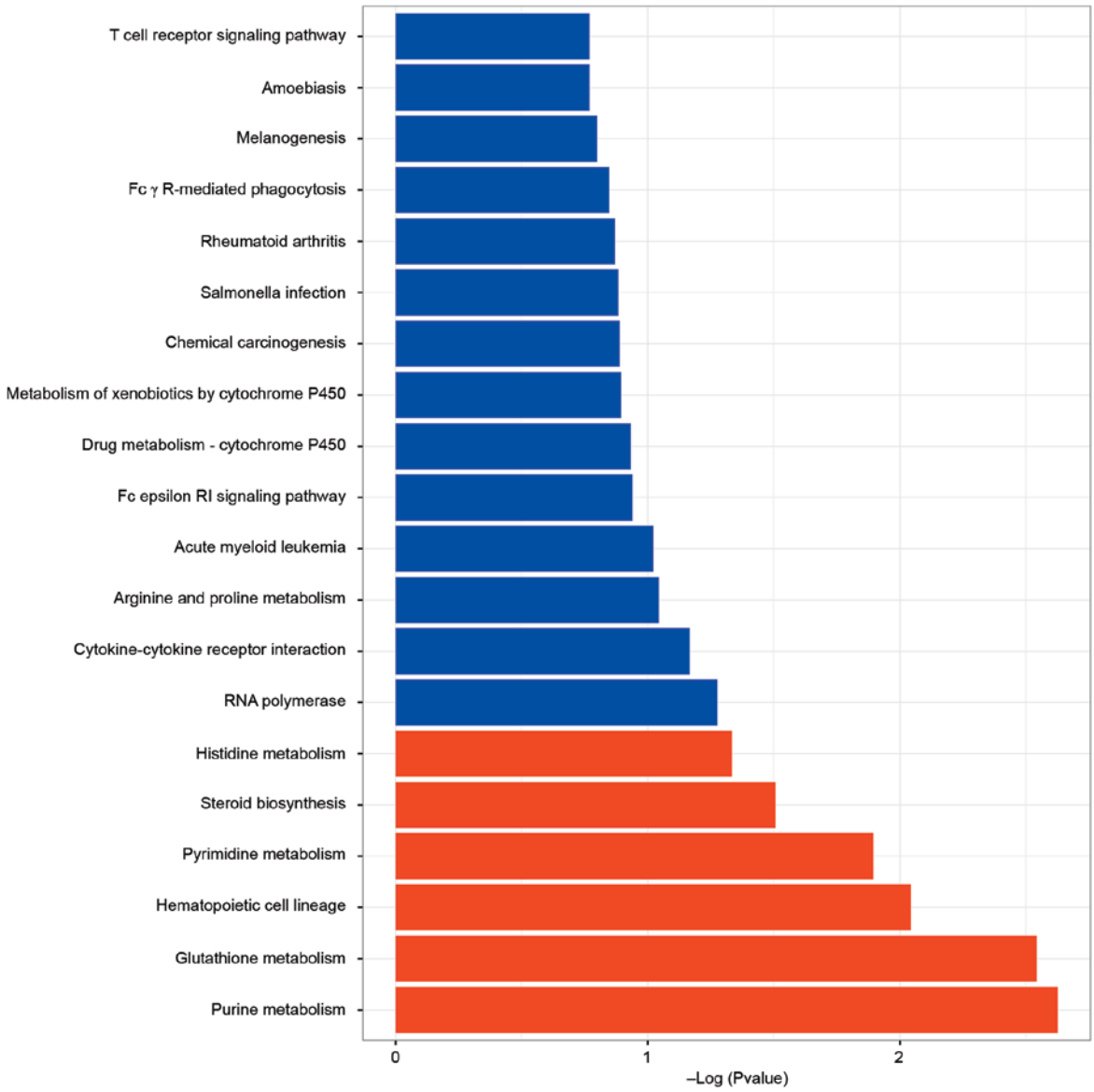

Figure 6. KEGG pathway analysis for differentially-expressed genes. The horizontal axis represents the -log (p-value) of the pathway and the vertical axis represents the pathway list. P<0.05 was used as the threshold for selecting the significant KEGG pathways (red bars). KEGG, Kyoto Encyclopedia of Genes and Genomes.

that binding of phosphorylated-Stat3 to the APLN promoter is the final step underlying proinflammatory cytokine-induced enteric APLN expression during intestinal inflammation. However, whether APLN is involved in the Stat5 signaling remains to be elucidated. It may be hypothesized that APLN affects T cells through the Stat5 signaling pathway; however, further studies are required.

Mast/stem cell growth factor receptor, also known as proto-oncogene c-Kit, tyrosine-protein kinase Kit, or cluster of differentiation 117 is a receptor tyrosine kinase protein that in humans is encoded by the KIT gene (42). The GO analysis results revealed that KIT was closely associated with tyrosine phosphorylation of Stat5 protein, thus indicating that dysregulation of Stat5 may be important in the process of SCAS.

Brain-type creatine kinase is a creatine kinase encoded by the CKB gene in humans, which is associated with creatine kinase activity and cellular monovalent inorganic anion homeostasis (43). However, to the best of our knowledge, the involvement of CKB in the process of SCAS has not yet been reported.

In conclusion, APLN and KIT may serve important roles in the MMF treatment of SCAS. The results of the present study suggested that MMF may upregulate APLN to inhibit the proliferation of T cells through the Stat5 signaling pathway. Further investigation of the function of APLN and KIT in atherosclerosis is urgently required.

Acknowledgements

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