

First-in-human immunomodulation mechanism research of chlorogenic acid in patients with recurrent high-grade glioma

Shoubo Yang^{1*}, Zhuang Kang¹, Ping Li², Lin Feng², Kaidi Gao³, Xiaosong Zhong⁴, Shujun Cheng^{2*}, Wenbin Li^{1*}

¹Department of Oncology, Beijing Shijitan Hospital, Capital Medical University, Beijing 100038, China; ²State Key Laboratory of Molecular Oncology, Department of Etiology and Carcinogenesis, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China; ³Beijing Rehabilitation Hospital of Capital Medical University, Beijing 100144, China; ⁴Central Laboratory, Beijing Shijitan Hospital, Capital Medical University, Beijing 100038, China

*These authors contributed equally to this work.

Correspondence to: Wenbin Li, MD. Department of Oncology, Beijing Shijitan Hospital, Capital Medical University, No. 10 Tieyi Road, Beijing 100038, China. Email: liwenbin@ccmu.edu.cn; Shujun Cheng, MD. State Key Laboratory of Molecular Oncology, Department of Etiology and Carcinogenesis, National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 17 Panjiayuan Nanli, Beijing 100021, China. Email: chengshj@cae.cn.

Abstract

Objective: Recurrent high-grade glioma presents the worst prognosis among brain tumors. Given that most high-grade gliomas recur after a standard surgery and radiochemotherapy, new treatment modalities are needed. Chlorogenic acid (CGA) displays regulatory, antitumor and immunoregulatory bioactivities in animals and cultured human tumor cells. This study aimed to explore the antitumor mechanism of CGA in patients with recurrent high-grade glioma by high-throughput RNA-sequencing (RNA-seq).

Methods: A case series with six patients was included. Their peripheral blood samples were collected two times (pretreatment and day 15) during CGA therapy. RNA-seq was used to sequence transcriptomes. The differentially expressed genes (DEGs) detected by MATLAB software were used to conduct Kyoto Encyclopedia of Genes and Genomes pathway and Gene Ontology functional analyses.

Results: Two patients (P3 and P4) achieved stable disease state after CGA therapy, while the tumors progressed in the other four patients. The progression-free survival times of P3 and P4 were 8.1 and 4.9 months, respectively. The overall survival time of both patients was 16.7 months, and these patients are still alive to date. P3 and P4 showed the least average fold changes in total DEGs among the six patients. A total of 269 and 182 commonly upregulated and downregulated DEGs were identified in P3 and P4, respectively. These genes were enriched with significant signal pathways or gene terms related to immune system. Gene expression levels from these pathways or terms were upregulated or downregulated uniformly in P3 and P4, which was significantly different from the results in other patients.

Conclusions: This study presented evidence about the immunomodulation function of CGA in patients with recurrent high-grade glioma. In CGA responsive patients, CGA may change the gene expression and upregulate the immune system activity to inhibit tumor development, thereby making it a potential antitumor drug.

Keywords: Chlorogenic acid; glioma; therapy; RNA-sequencing

Submitted Jan 31, 2018. Accepted for publication Apr 27, 2018.

doi:

View this article at: <https://doi.org/>

Introduction

High-grade glioma, including glioblastoma multiforme and anaplastic astrocytoma, still presents poor prognosis after standard therapy [surgical resection and concurrent radiochemotherapy with subsequent adjuvant temozolomide (TMZ)] (1). Tumor recurs inevitably in almost all patients. This standard of care cannot be used for recurrent or TMZ-resistant high-grade gliomas. Antiangiogenic drugs, such as bevacizumab, result in no improvement on the overall survival (OS) of patients with glioblastoma (2). Despite the increasing number of new therapy strategies in the fields of immunotherapy and targeted therapy, effective therapies are still urgently needed (3-5).

Chlorogenic acid (CGA), as one of the most available polyphenols in food (such as tea and coffee), is gaining increasing attention (6). A large number of studies focus on the multifunctional properties of CGA, such as its antitumor, neuroprotective, anti-inflammatory, antioxidant, antiobesity, and free radical scavenger activities (7-13). However, the antitumor functional mechanisms of CGA, including immunomodulation, antiangiogenesis, and apoptosis induction, relatively differ in existing reports (14-16).

RNA sequencing (RNA-seq) has been extensively applied to analyze the continuously changing cellular transcriptome by using next-generation sequencing. In comparison with hybridization-based microarrays, RNA-seq facilitates the ability to assess the alternative gene spliced transcripts, post-transcriptional modifications, gene fusions, mutations, and changes in gene expression over time or differences in gene expression in different groups or treatments (17). Thus, in the present study, we aimed to explore the antitumor immunomodulation mechanism of CGA by using RNA-seq.

Materials and methods

Patients and study protocol

A case series of patients receiving CGA treatment in Beijing Shijitan Hospital was enrolled in our study from April 2016 to September 2016. This study was approved by the ethics committee of Beijing Shijitan Hospital. All patients were required to sign an informed consent before the enrollment. Patients were eligible for participation in the study only if their tumor recurred after standard care for advanced glioma (grades III-IV). Patients were

excluded if they received chemotherapy or radical radiotherapy within 4 weeks prior to enrollment. Eligible patients received daily CGA injection in a cycle of one month. Adverse events were assessed by the Common Terminology Criteria for Adverse Events version 4.0. Response Assessment in Neuro-Oncology (RANO) Criteria was used to determine the disease state based on imaging assessment [magnetic resonance imaging (MRI)], clinical symptom, and glucocorticoid use. Progression-free survival (PFS) was defined as the time from enrollment until progression or death, whichever occurred first. OS was measured from enrollment until death from any cause. Assessments were carried out at the baseline, after cycles 1 and 2, every 2 months throughout the maintenance phase, and at the time of disease progression.

Total RNA extraction

Blood sample (5-6 mL) was collected between 7 am and 8 am from cubital veins and placed in ethylenediamine-tetraacetic acid-containing tubes before treatment and on day 15 of treatment with CGA. RNA was extracted from the peripheral blood leukocyte cells of patients. Briefly, plasma and blood cells were separated by centrifugation at 120 g for 20 min. Additionally, 2 mL of blood cells were mixed with 8 mL of erythrocyte lysis buffer (Qiagen GmbH, Hilden, Germany) and incubated for 15 min on ice with gentle agitation. Following centrifugation at 400 g for 10 min, pelleted cells were washed once with 1 mL of erythrocyte lysis buffer. Cell pellet was subsequently fully resuspended in 1 mL of TRIzol reagent (Invitrogen, Paisley, UK). RNA was extracted according to the instructions of Qiagen RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany).

RNA quantification and qualification

First, agarose gel electrophoresis was used to monitor RNA degradation and contamination on 1% agarose gels. Second, the RNA purity, as shown by OD_{260/280} and OD_{260/230}, was evaluated by NanoDrop 1000 spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the exact RNA integrity number (RIN) of each sample was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent, Palo Alto, CA, USA). In accordance with the protocols, samples with qualified concentration, purity and RIN can be used in cDNA library building.

cDNA library preparation and sequencing

Strand-specific cDNA libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) following the manufacturer's protocols. Briefly, mRNA was isolated and fragmented from the total RNA in NEBNext First Strand Synthesis Reaction Buffer. Afterward, first-strand cDNA synthesis was performed by adding murine RNase inhibitor and ProtoScript II reverse transcriptase to primed mRNA. Second-strand synthesis reaction buffer and enzyme mix were used to carry out the second-strand synthesis. End repair, adaptor ligation, and double-stranded cDNA purification were conducted in sequence. Finally, the cDNA library was enriched by polymerase chain reaction (PCR). Purification with AMPure XP beads was performed three times in the whole reaction: after the second-strand synthesis, after the ligation reaction, and after PCR. After determining the quality by using the Bioanalyzer 2100 system, the libraries were sequenced using the Illumina HiSeq 150 instrument (Illumina, San Diego, CA, USA).

Quantification and gene expression level

Clean reads were obtained to perform downstream analyses by removing low-quality reads from raw reads. The hg19 human reference genome was downloaded from the National Center for Biotechnology Information (ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/). The reference genome index was established by using HISAT2 version 2.0.5. Paired-end clean reads were aligned to the reference genome by using HISAT2. FeatureCounts version 1.6.1 software was used to count the reads mapped to each gene. The fragments per kilobase of transcript sequence per millions (FPKM) of each gene were calculated on the basis of sequencing depth and gene length. The Log_2 (FPKM) value of each gene was used for downstream analysis.

Differential expression analysis and functional annotation

Differential expression analysis on each patient before treatment and after 15 d of treatment with CGA was performed using MATLAB version 7.0 software. Paired Student's *t*-test was used to analyze paired samples. Genes with a P value of <0.05 found by MATLAB were assigned as differentially expressed. The fold change (FC) threshold of 2 was used to screen targeted genes. These genes are collected to conduct the following functional annotation to make clear how their network make an impact. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database that stores information on how molecules and genes are networked (18). Gene Ontology (GO) analysis is applied in functional studies of large-scale genomic or transcriptomic data (19). To perform KEGG pathway and GO functional analyses, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>). DAVID is an online bioinformatics tool used to perform functional interpretation of large lists of genes or proteins (20). After a gene list was imported to the DAVID, enriched pathways and terms were screened by P values <0.05 according to the protocol (21). Through these specified pathways and terms, significant genes can be identified.

Results

Patients

With regard to the immunomodulation mechanism research of CGA, the blood samples from the enrolled six patients were analyzed by high throughput RNA-seq. Baseline information is shown in *Table 1*. The first cycle of CGA therapy in patients P3 and P4 caused the lesions to shrink, thereby achieving stable disease state according to the RANO standard (*Figure 1*). Accordingly, these patients received four and five cycles of CGA injection until disease

Table 1 Baseline demographic and clinical characteristics

Patients	Age (year)	Sex	Grade	Dosage (mg/kg)	Outcome	PFS (month)	OS (month)
P1	27	Male	IV	2	PD	1.0	2.1
P2	32	Female	IV	2	PD	1.0	7.6
P3	48	Female	IV	3	SD	8.1	Alive
P4	22	Male	III-IV	3	SD	4.9	Alive
P5	49	Male	IV	3	PD	1.0	6.3
P6	43	Male	III-IV	4	PD	1.0	7.4

PFS, progression-free survival; OS, overall survival; PD, progress of disease; SD, stable disease.

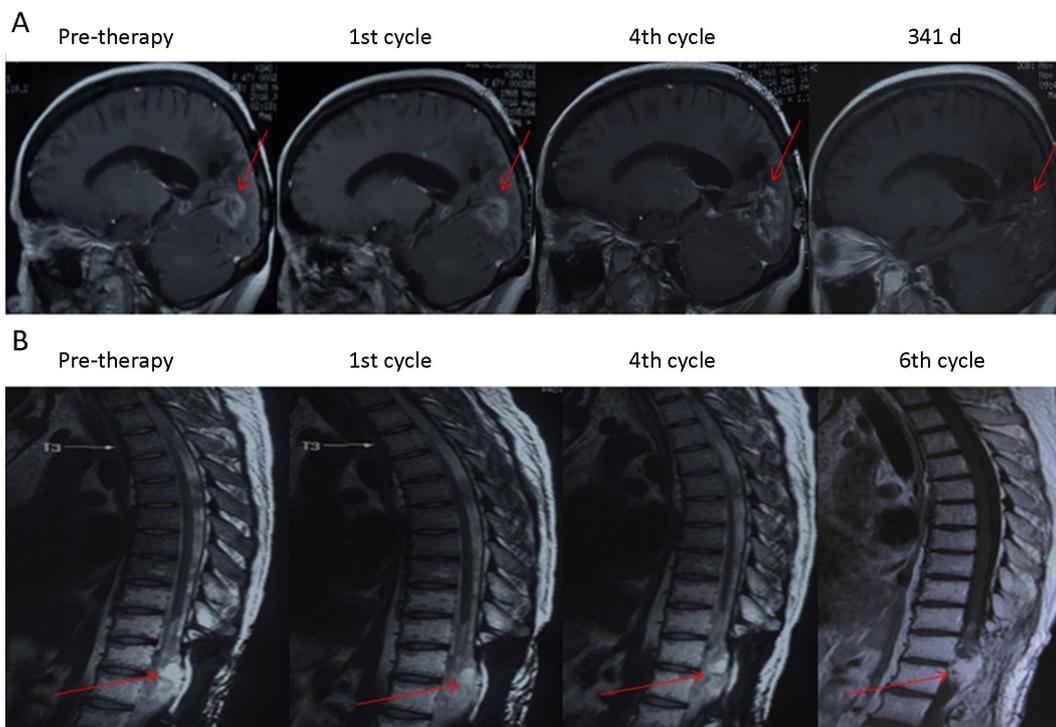


Figure 1 Magnetic resonance imaging (MRI) images before and after chlorogenic acid (CGA) therapy in patients P3 and P4. (A) For patient P3, brain MRI images are shown pre-therapy, i.e., post tumor recurrence and prior to CGA administration, as well as following 1st cycle and 4th cycle, and 341 d after the last CGA treatment; (B) For patient P4, thoracic vertebral MRI images are shown pre-therapy, as well as following 1st cycle, 4th cycle and 6th cycle. Red arrows indicate sites of tumors in patients.

progression, and their PFS was 8.1 and 4.9 months, respectively. After tumor progression, P3 have received TMZ chemotherapy again for 12 cycles until now, and P4 underwent third surgery. These patients both survived for more than 16.5 months after CGA treatment, and they are still alive to date. Their medical histories are shown in [Figure 2](#). Patients P1, P2, P5, and P6 showed no inhibited tumor progression after CGA therapy.

Differential expression analysis

Qualified RNA was extracted successfully from all samples of six patients for sequencing. The quality control results are presented in the supplementary files ([Supplementary Figure S1, S2](#)). Given that the six patients displayed different CGA therapy efficacies, we divided them into responsive (P3 and P4) and nonresponsive (P1, P2, P5, and P6) subgroups to facilitate downstream analyses. All significantly upregulated and downregulated genes ($P < 0.05$, $FC > 2$) were counted in these patients from pretherapy to day 15. The number of differentially expressed genes (DEGs) in six patients ranged from 1,500 to 1,875, with the mean number of 1,655. [Figure 3](#) shows the comprehensive

expression of DEGs in six patients via the FC values of each gene. As shown in [Figure 4](#), the average FC values of P1, P3, and P4 were significantly less than those of P2, P5, and P6.

KEGG pathway and GO functional analyses

A total of 269 and 182 common upregulated and downregulated genes, respectively, were found in P3 and P4. Several of the most highly upregulated DEGs, such as *CD52* (CAMPATH-1 antigen), *APOBEC3C* (DNA dC->dU-editing enzyme APOBEC-3C), and histone H2B type 2-E (*HIST2H2BE*), are involved in immune cells or innate immune response (22). The most highly repressed DEGs include tumor necrosis factor receptor superfamily member 10C (*TNFRSF10C*), which acts as a receptor for the cytotoxic ligand TRAIL; GTP activity-related proteins, such as Rho GTPase-activating protein 30 (*ARHGAP30*), ras-related C3 botulinum toxin substrate 1 (*RAC1*) and tubulin-specific chaperone C (23,24); and an intracellular lectin protein, vesicular integral-membrane protein VIP36 (*LMAN2*).

These upregulated genes were enriched in nine KEGG

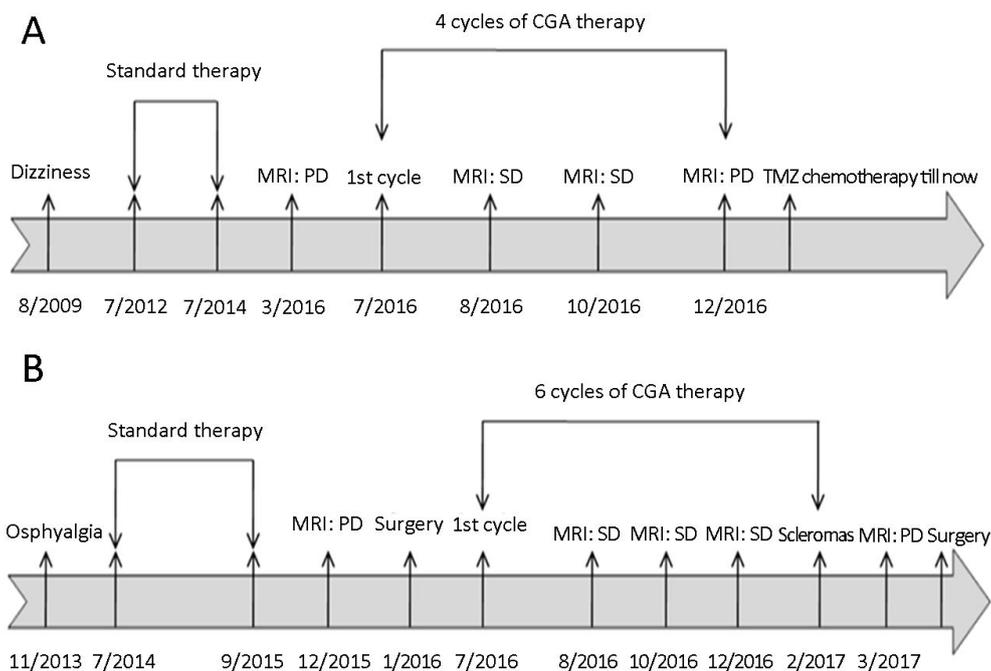


Figure 2 Treatment pipelines of patients P3 and P4. (A) Patient P3 was diagnosed with primary glioblastoma multiform (GBM) in 2012 and tumor progressed after standard care in 2016. Chlorogenic acid (CGA) treatment inhibited tumor development in prior 3 cycles. After the second progression, temozolomide (TMZ) therapy was used till now; (B) Patient P4 received two surgeries before enrollment, with pathological diagnosis of astrocytoma to anaplastic astrocytoma (WHO II to WHO III-IV). Skin scleromas occurred in the 6th cycle of CGA therapy caused by injection and treatment was suspended. He received the 3rd surgery after progress of disease (PD) and no more chemotherapy till now. SD, stable of disease.

pathways with $P < 0.05$. The Janus kinase and signal transducer and activator of transcription (Jak-STAT) signaling was the most important pathway that participated in immune system regulation. Pathways enriched by downregulated genes included cytokine-cytokine receptor interaction and NF-kappa B signaling pathway, which were both active in a series of gene expression, such as interleukin 8 (*IL-8*), *SDF-1 α* , *BAFF* and *ICAM*, to influence the survival, lymphopoiesis, development, and adhesion of immune cells. More than 100 GO terms were collected by upregulated or downregulated genes with $P < 0.05$. Among these terms, 14 and 10 GO terms were related with immunomodulation, respectively, and they were involved in lymphocyte differentiation, aggregation, and immune response (Figure 5).

Significant genes from KEGG pathways and GO terms

Ten DEGs were identified from the Jak-STAT signaling pathway. Several of these DEGs were involved in IL signaling pathway: *CSF2R $\beta\beta$* (cytokine receptor common

subunit beta), which is a high-affinity receptor for IL-3 and IL-5; signal transducer and activator of transcription 4, which plays a role in IL-12 signaling; IL-13 receptor subunit alpha-1, which binds with low-affinity to IL-13; and interferon gamma receptor 2, which is involved with IL-10 receptor activity (25). Nine DEGs were identified from cytokine-cytokine receptor interaction and NF-kappa B signaling pathway. Among these genes, Granulocyte colony-stimulating factor receptor (*CSF3R*) is a receptor for granulocyte colony-stimulating factor and essential for granulocytic maturation (26); IL-10 receptor subunit alpha and IL-10 receptor subunit beta act as receptors for IL-10 (27); lymphotoxin-beta plays a specific role in immune response regulation; and platelet factor 4 is chemotactic for neutrophils and monocytes (28). A total of 46 DEGs were identified from upregulated genes enriched with 14 GO terms. These DEGs also included *APOBEC3C* and *HIST2H2BE*. Several DEGs, especially T cells, such as HLA class II histocompatibility antigen-DRB1-13 β chain, Zn finger and BTB domain-containing protein 7B, and tumor necrosis factor ligand superfamily member 8, are

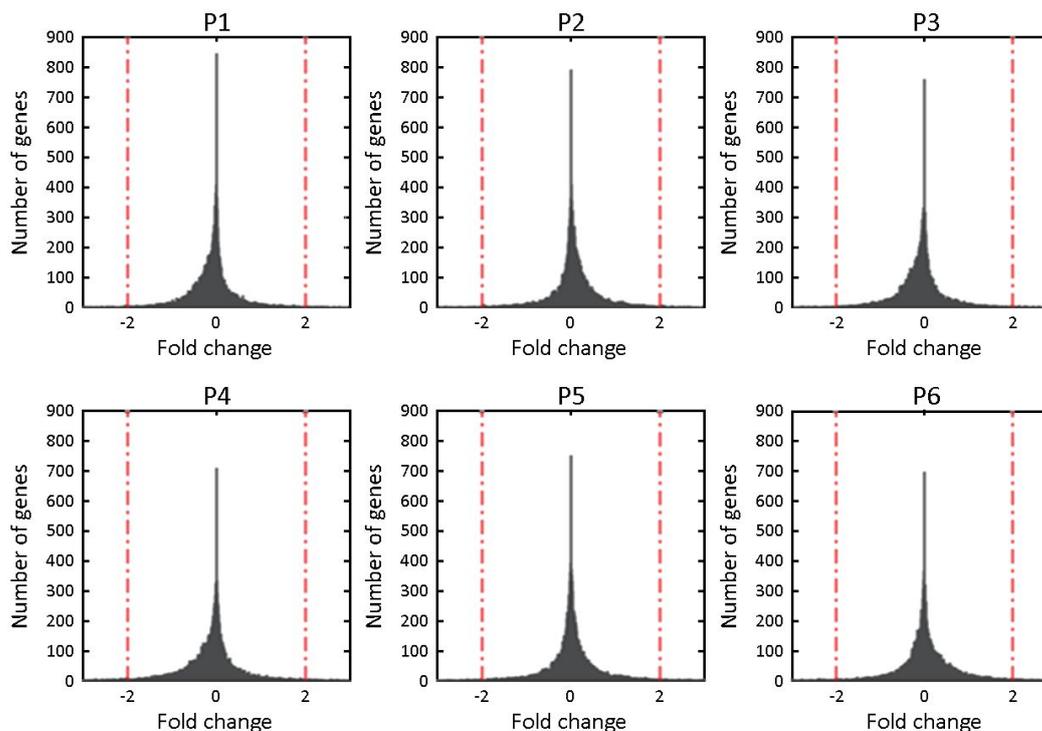


Figure 3 Number of differentially expressed genes by fold change in each patient. The number of differentially expressed genes in six patients ranged from 1,500 to 1,875, with the mean number of 1,655. Downregulated differentially expressed genes were more than upregulated ones in P1, P3 and P4.

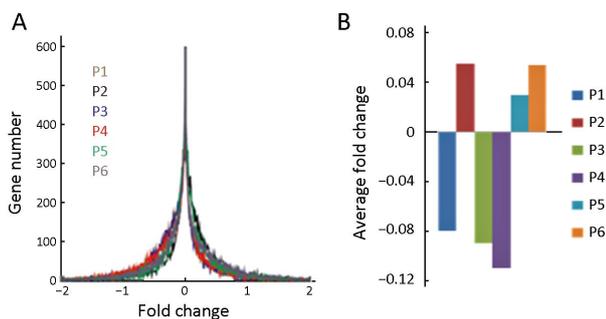


Figure 4 Analysis of differentially expressed genes of 6 patients. (A) Fold changes of differentially expressed genes in 6 patients in one plot; (B) Difference of average fold change among 6 patients. P3 and P4 had the least average fold change.

correlated with leukocyte activation and aggregation (29). A total of 30 DEGs were identified from downregulated genes enriched with 10 GO terms. Several DEGs, including C5a anaphylatoxin chemotactic receptor 2, cytoplasmic FMR1-interacting protein 1 and NCK-associated protein 1-like, also participated in immune response (30-32). Other DEGs, such as *CSF3R*, *SLC7A7* (Y+L amino acid transporter 1), and caspase recruitment

domain-containing protein 9, can regulate leukocyte migration (33,34). As shown in *Figure 6*, the expression levels of all these genes were upregulated or downregulated uniformly in P3 and P4, which was significantly different from the expression in other patients (*Supplementary Table S1*).

Discussion

This study is to explore the efficacy of CGA in patients with high-grade glioma. To investigate about the antitumor mechanism of CGA, we used RNA-seq to characterize differences in the gene expression of peripheral blood leukocytes from the six patients before and after CGA treatment. Our results indicated that CGA may influence the gene expression and immune system functions of patients, thereby inhibiting tumor development.

Growing concern regarding the negative consequences of cytotoxic chemicals has caused increasing attention toward natural and herbal substances. Phenolic acids are one of these groups that received increasing attention because of their many promising effects related to their

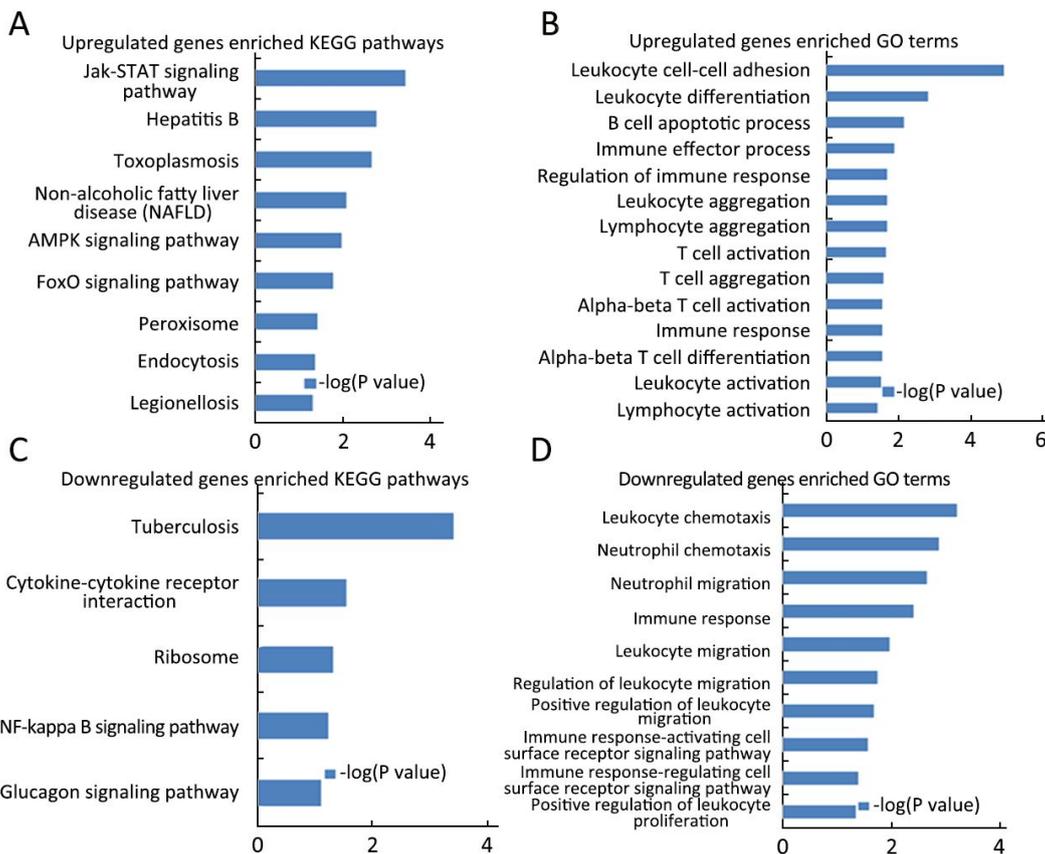


Figure 5 Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms. (A) Jak-STAT signaling pathway was the most important one in 9 enriched pathways and took part in regulation of immune system; (B) 14 GO terms were related to immunomodulation, including leukocytes differentiation, activation, aggregation, and immune response; (C) Pathways enriched by downregulated genes included cytokine-cytokine receptor interaction and NF-kappa B signaling pathway, which can influence survival, lymphopoiesis, development and adhesion of immune cells; (D) 10 GO terms were related to immunomodulation, involved with lymphocytes migration, proliferation, and immune response.

anti-inflammatory and antioxidant properties; CGA is also a primary example because of its antitumor effects in cultured cancer cells and animal xenografts (35,36). For instance, in U87 glioma cells, CGA can regulate their invasive phenotype through the inhibition of MMP-2 secretion and migration with or without sphingosine-1-phosphate induction (37). *Leonurus sibiricus* L. (Lamiaceae) is rich in polyphenolic compounds, such as CGA, caffeic acid, and ferulic acid. Root extracts of *L. sibiricus* L. can cause significant cell growth inhibition, S- and G2/M-phase cell cycle arrest, and apoptosis by changing the Bax/Bcl-2 ratio and p53 activation in the treatment of human glioblastoma cells (38). Recurrent high-grade gliomas are associated with considerably poor clinical outcomes. Hence, we evaluated CGA in our patients.

In the present study, the mean PFS and OS for

responsive patients P3 and P4 were 6.5 and 16.5 months, respectively. CGA prolonged their survival times significantly either in PFS or OS compared with those in previous reports (39-41). Furthermore, two responsive patients (P3 and P4) are still alive to date, and they both received CGA injections of 3 mg/kg dosage. Thus, the CGA dosage level may play a role in the clinical outcome of patients. Moreover, patient P3 was TMZ-resistant in the state of first tumor progression. Notably, she received TMZ chemotherapy again, which resulted in a remarkable effect after CGA treatment. This result was similar to the findings in our preclinical studies. We evaluated the efficacy of CGA in subcutaneous and orthotopic mouse xenograft model established by injecting G422 glioma cells. Tumor progression was inhibited, with inhibitory rates between 48% and 50%. CGA combined with TMZ can

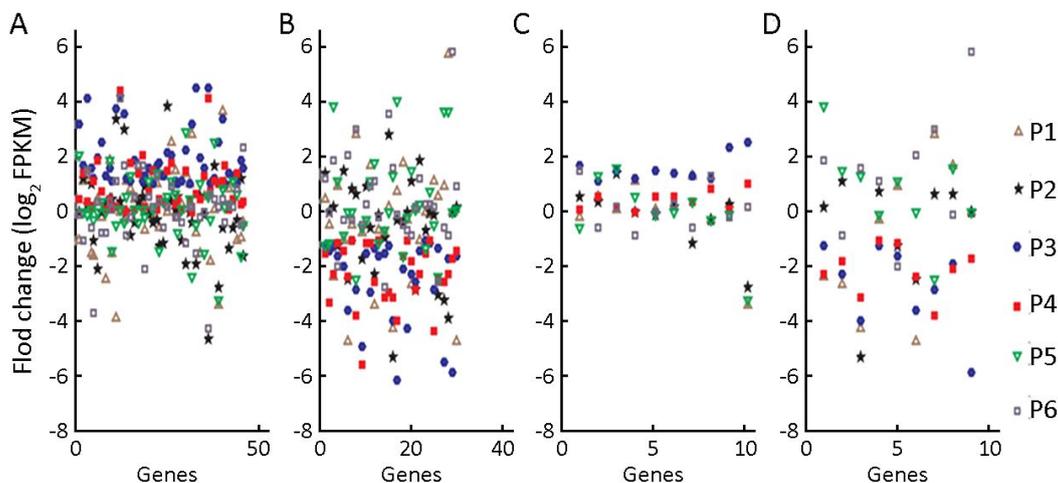


Figure 6 Expressions of 95 genes from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms in 6 patients. (A) Forty-six genes from GO terms were upregulated uniformly in P3 and P4; (B) 30 genes from GO terms were downregulated uniformly in P3 and P4; (C) 10 genes from KEGG pathways were upregulated uniformly in P3 and P4; (D) 9 genes from KEGG pathways were downregulated uniformly in P3 and P4.

increase this inhibitory rate to 70%. This effect may be due to the fact that CGA and TMZ exert synergistic effects on antitumor functions.

Given that the control arm was unassigned in this study, we found differential gene expression between responsive and nonresponsive patients. Patients P3 and P4 showed the least average FC of total DEGs in six patients. This result suggested that CGA may affect the gene expression levels of peripheral blood leucocytes in these two patients to achieve disease inhibition. To understand the biological effects of these gene expression changes, 269 and 182 common upregulated and downregulated genes were identified, respectively. Several of the most highly upregulated DEGs are involved in immune cells or innate immune response (*CD52*, *APOBEC3C*, and *HIST2H2BE*), and most highly repressed DEGs play a role in cytotoxic ligand receptor and GTP activity (*TNFRSF10C*, *ARHGAP30*, and *RAC1*).

GO functional and KEGG pathway analyses were performed to determine how DEGs work together. Upregulated DEGs were enriched in Jak-STAT signaling pathway, which was consistent with the result of our previous study. CGA inhibits glioblastoma growth through repolarizing macrophage from M2 to M1 phenotype via the promotion of STAT1 activation and inhibition of STAT6 activation *in vitro* and *in vivo* (42). Enriched GO terms were related with a series of vital processes of immune regulation, including proliferation, differentiation, migration, activation, aggregation, and immune response.

Similarly, a study about the mechanism of CGA antitumor in BALB/c mice demonstrated that *CaN*, *NFATC2*, *NFATC2ip*, and *NFATC3* can be upregulated by CGA, thereby causing improvement in the immune factors, such as IL-2R and interferon- γ . Finally, nature killer cells, T cells, and macrophages are promoted by activation and proliferation (43). Moreover, genes from GO terms and KEGG pathways were regulated to the same direction (upregulated or downregulated) after CGA treatment in patients P3 and P4. This result indicated the improved immune anticancer activity and survival of these two patients.

In a randomized phase 2 trial using axitinib in patients with recurrent glioblastoma, results suggested that axitinib can promote response rate and PFS compared with historical controls (44). Further study reported that although axitinib is a vascular endothelial growth factor receptor inhibitor, it can also regulate immunosuppression derived from tumor. Treatment with axitinib can promote proliferation of naive and central memory T cells. However, most patients treated with axitinib show progression in the long run, and a high lymphocyte activation gene 3 (*LAG3*) protein expression may be correlated with disease progression (45). *LAG3*, which is expressed on exhausted T cells, inhibits their antitumor activity similar to programmed cell death protein 1 (46). Consistently, in our study, *LAG3* was downregulated more significantly in P3 and P4 than those in others, with the FC values of -1.25 and -1.86 , respectively (-1.23 , 1.49 , -1.09 ,

and 1.04 in P1, P2, P5, and P6, respectively). This result also suggested that the immune activities of P3 and P4 were enhanced by CGA, thereby prolonging their survival times.

Although this study provided important evidence to further understand the antitumor immunomodulation mechanism of CGA, non-negligible limitations still exist. In this study, the sample size was small, and no control group was assigned. These disadvantages should be improved in subsequent study.

Conclusions

This study is to explore the immunomodulation mechanism of CGA in patients with recurrent high-grade glioma. Results showed that CGA may change gene expression levels to upregulate the immune system activity in responsive patients. Future study is necessary to further understand the functional mechanism of CGA.

Acknowledgements

We thank Prof. Webster Cavenne (Ludwig Institute for Cancer Research, La Jolla, United States) for manuscript review support; Prof. Jinzhi Lei (Zhou Pei-Yuan Center for Applied Mathematics, Beijing, China) for data analysis; and Prof. Jianbo Sun (Hospital of Stomatology of Sun Yat-sen University, Guangzhou, China) for manuscript review support.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987-96.
2. Lombardi G, Pambuku A, Bellu L, et al. Effectiveness of antiangiogenic drugs in glioblastoma patients: A systematic review and meta-analysis of randomized clinical trials. *Crit Rev Oncol Hematol* 2017;111:94-102.
3. Kreisl TN, Lassman AB, Mischel PS, et al. A pilot study of everolimus and gefitinib in the treatment of recurrent glioblastoma (GBM). *J Neurooncol* 2009;92:99-105.
4. Hunn MK, Bauer E, Wood CE, et al. Dendritic cell vaccination combined with temozolomide retreatment: results of a phase I trial in patients with recurrent glioblastoma multiforme. *J Neurooncol* 2015;121:319-29.
5. Schijns VE, Pretto C, Devillers L, et al. First clinical results of a personalized immunotherapeutic vaccine against recurrent, incompletely resected, treatment-resistant glioblastoma multiforme (GBM) tumors, based on combined allo- and auto-immune tumor reactivity. *Vaccine* 2015;33:2690-6.
6. Meng S, Cao J, Feng Q, et al. Roles of chlorogenic acid on regulating glucose and lipids metabolism: A review. *Evid Based Complement Alternat Med* 2013; 2013:801457.
7. Yan Y, Li J, Han J, et al. Chlorogenic acid enhances the effects of 5-fluorouracil in human hepatocellular carcinoma cells through the inhibition of extracellular signal-regulated kinases. *Anticancer Drugs* 2015; 26:540-6.
8. Heitman E, Ingram DK. Cognitive and neuroprotective effects of chlorogenic acid. *Nutr Neurosci* 2017;20:32-9.
9. Hao ML, Pan N, Zhang QH, et al. Therapeutic efficacy of chlorogenic acid on cadmium-induced oxidative neuropathy in a murine model. *Exp Ther Med* 2015;9:1887-94.
10. Liang N, Kitts DD. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients* 2016;8:pii:e16.
11. Puupponen-Pimiä R, Nohynek L, Meier C, et al. Antimicrobial properties of phenolic compounds from berries. *J Appl Microbiol* 2001;90:494-507.
12. Cho AS, Jeon SM, Kim MJ, et al. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food Chem Toxicol* 2010;48:937-43.
13. Saidi Merzouk A, Hafida M, Medjdoub A, et al. Alterations of hepatocyte function with free radical generators and reparation or prevention with coffee polyphenols. *Free Radic Res* 2017;51:294-305.
14. Kang TY, Yang HR, Zhang J, et al. The studies of chlorogenic acid antitumor mechanism by gene chip detection: the immune pathway gene expression. *J Anal Methods Chem* 2013;2013:617243.

15. Park JJ, Hwang SJ, Park J-H, et al. Chlorogenic acid inhibits hypoxia-induced angiogenesis via down-regulation of the HIF-1 α /AKT pathway. *Cell Oncol* 2015;38:111-8.
16. Liu YJ, Zhou CY, Qiu CH, et al. Chlorogenic acid induced apoptosis and inhibition of proliferation in human acute promyelocytic leukemia HL-60 cells. *Mol Med Rep* 2013;8:1106-10.
17. Maher CA, Kumar-Sinha C, Cao X, et al. Transcriptome sequencing to detect gene fusions in cancer. *Nature* 2009;458:97-101.
18. Ogata H, Goto S, Sato K, et al. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 1999;27:29-34.
19. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000;25:25-9.
20. Jiao X, Sherman BT, Huang da W, et al. DAVID-WS: a stateful web service to facilitate gene/protein list analysis. *Bioinformatics* 2012;28:1805-6.
21. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2008;4:44-57.
22. Yu Q, Chen D, König R, et al. APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication. *J Biol Chem* 2004;279:53379-86.
23. Naji L, Pacholsky D, Aspenstrom P. ARHGAP30 is a Wrch-1-interacting protein involved in actin dynamics and cell adhesion. *Biochem Biophys Res Commun* 2011;409:96-102.
24. Bartolini F, Bhamidipati A, Thomas S, et al. Functional overlap between retinitis pigmentosa 2 protein and the tubulin-specific chaperone cofactor C. *J Biol Chem* 2002;277:14629-34.
25. Soh J, Donnelly RJ, Kotenko S, et al. Identification and sequence of an accessory factor required for activation of the human interferon γ receptor. *Cell* 1994;76:793-802.
26. Dong F, Hoefsloot LH, Schelen AM, et al. Identification of a nonsense mutation in the granulocyte-colony-stimulating factor receptor in severe congenital neutropenia. *Proc Natl Acad Sci U S A* 1994;91:4480-4.
27. Donnelly RP, Sheikh F, Kotenko SV, et al. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J Leukoc Biol* 2004;76:314-21.
28. Gupta SK, Hassel T, Singh JP. A potent inhibitor of endothelial cell proliferation is generated by proteolytic cleavage of the chemokine platelet factor 4. *Proc Natl Acad Sci U S A* 1995;92:7799-803.
29. Widom RL, Culic I, Lee JY, et al. Cloning and characterization of hcKrox, a transcriptional regulator of extracellular matrix gene expression. *Gene* 1997;198:407-20.
30. Park H, Chan MM, Iritani BM. Hem-1: putting the "WAVE" into actin polymerization during an immune response. *FEBS Lett* 2010;584:4923-32.
31. Cain SA, Monk PN. The orphan receptor C5L2 has high affinity binding sites for complement fragments C5a and C5a des-Arg(74). *J Biol Chem* 2002;277:7165-9.
32. Kobayashi K, Kuroda S, Fukata M, et al. p140Sra-1 (specifically Rac1-associated protein) is a novel specific target for Rac1 small GTPase. *J Biol Chem* 1998;273:291-5.
33. Bertin J, Guo Y, Wang L, et al. CARD9 is a novel caspase recruitment domain-containing protein that interacts with BCL10/CLAP and activates NF-kappa B. *J Biol Chem* 2000;275:41082-6.
34. Torrents D, Estévez R, Pineda M, et al. Identification and characterization of a membrane protein (γ +L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity γ +L. A candidate gene for lysinuric protein intolerance. *J Biol Chem* 1998;273:32437-45.
35. Santana-Gálvez J, Cisneros-Zevallos L, Jacobo-Velázquez DA. Chlorogenic acid: recent advances on its dual role as a food additive and a nutraceutical against metabolic syndrome. *Molecules* 2017;22:pii:e358.
36. Venditti A, Frezza C, Bianco A, et al. Polar constituents, essential oil and antioxidant activity of marsh woundwort (*Stachys palustris* L.). *Chem Biodivers* 2017;14.
37. Belkaid A, Currie JC, Desgagnés J, et al. The chemopreventive properties of chlorogenic acid reveal a potential new role for the microsomal glucose-6-phosphate translocase in brain tumor progression.

- Cancer Cell Int 2006;6:7.
38. Sitarek P, Skala E, Toma M, et al. A preliminary study of apoptosis induction in glioma cells via alteration of the Bax/Bcl-2-p53 axis by transformed and non-transformed root extracts of *Leonurus sibiricus* L. *Tumour Biol* 2016;37:8753-64.
 39. Weller M, Tabatabai G, Kästner B, et al. MGMT promoter methylation is a strong prognostic biomarker for benefit from dose-intensified temozolomide rechallenge in progressive glioblastoma: the DIRECTOR trial. *Clin Cancer Res* 2015;21:2057-64.
 40. Field KM, Simes J, Nowak AK, et al. Randomized phase 2 study of carboplatin and bevacizumab in recurrent glioblastoma. *Neuro Oncol* 2015;17:1504-13.
 41. Taal W, Oosterkamp HM, Walenkamp AM, et al. Single-agent bevacizumab or lomustine versus a combination of bevacizumab plus lomustine in patients with recurrent glioblastoma (BELOB trial): a randomised controlled phase 2 trial. *Lancet Oncol* 2014;15:943-53.
 42. Xue N, Zhou Q, Ji M, et al. Chlorogenic acid inhibits glioblastoma growth through repolarizing macrophage from M2 to M1 phenotype. *Sci Rep* 2017;7:39011.
 43. Boulay F, Tardif M, Brouchon L, et al. Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. *Biochem Biophys Res Commun* 1990;168:1103-9.
 44. Duerinck J, Du Four S, Bouttens F, et al. Randomized phase II trial comparing axitinib with the combination of axitinib and lomustine in patients with recurrent glioblastoma. *J Neurooncol* 2018;136:115-25.
 45. Du Four S, Maenhout SK, Benteyn D, et al. Disease progression in recurrent glioblastoma patients treated with the VEGFR inhibitor axitinib is associated with increased regulatory T cell numbers and T cell exhaustion. *Cancer Immunol Immunother* 2016; 65:727-40.
 46. Nguyen LT, Ohashi PS. Clinical blockade of PD1 and LAG3--potential mechanisms of action. *Nat Rev Immunol* 2015;15:45-56.

Cite this article as: Yang S, Kang Z, Li P, Feng L, Gao K, Zhong X, Cheng S, Li W. First-in-human immunomodulation mechanism research of chlorogenic acid in patients with recurrent high-grade glioma. *Chin J Cancer Res* 2018

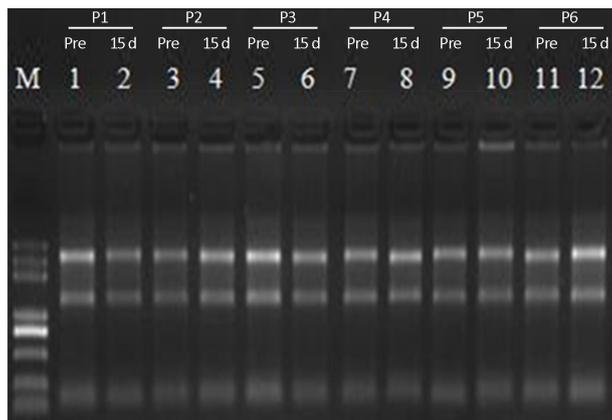
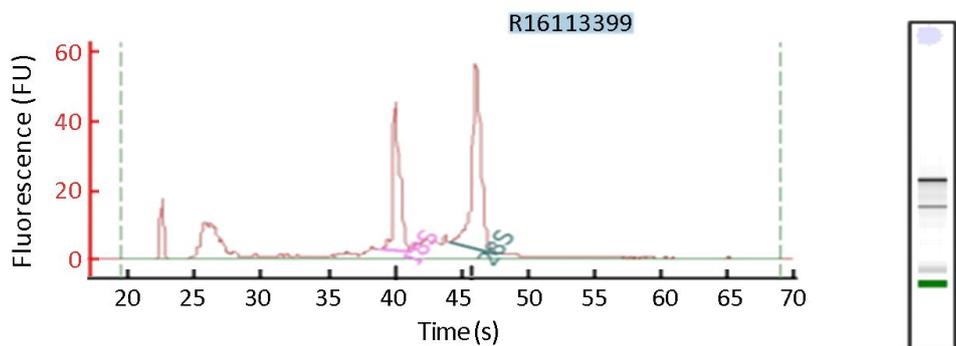


Figure S1 Photograph of agarose gel electrophoresis result. A total of 12 columns corresponding to samples pre-therapy and on the 15th day during chlorogenic acid (CGA) therapy from patients P1 to P6, respectively. Bright bands at 28S, 18S and 5S show purity of RNA. M, Trans 2K Plus marker.



Overall Result for sample 1:

RNA area: 344.2
 RNA concentration: 147 ng/ μ L
 rRNA ratio (28s/18s): 1.5
 RNA integrity number (RIN): 9.2 (B.02.08)
 Result flagging color:
 Result flagging label: RIN: 9.20

Fragment table for sample 1:

Name	Start Time (s)	End Time (s)	Area	% of total Area
18S	38.90	41.15	61.1	17.8
28S	44.10	47.65	92.8	26.9

Figure S2 Quality results with Agilent 2100 Bioanalyzer in one sample. The peak figure, concentration of RNA and RNA integrity number were showed.

Table S1 Upregulated or downregulated genes in KEGG pathways or GO terms

Expression changes	Gene name
Upregulated genes in KEGG pathways	<i>SOCS4, CSF2RB, EPOR, IFNGR2, IL13RA1, PIM1, PIK3CG, STAT4, STAT5A, PIAS1</i>
Downregulated genes in KEGG pathways	<i>CSF3R, IL10RA, IL10RB, LTB, PF4, BCL2, CCL4, CCR2, UBE2I</i>
Downregulated genes in GO terms	<i>IFI30, TRIM38, CRIP1, CSF3R, FCGR2A, SBNO2, CYFIP1, PIP5K1C, C5AR2, CD274, NCKAP1L, HLA-DPA1, IFI16, IL10RB, IL16, LTB, PF4, PRKACA, RAC1, BCL2, CCL4, CARD9, CCR2, CAMK2G, CRACR2A, APOL1, TNFRSF10C, SLC7A7, CD7, CD22</i>
Upregulated genes in GO terms	<i>BCL2L11, NDRG1, CLC, NFAM1, DDX3X, FKBP1A, ACIN1, FLT3LG, FOS, BLOC1S6, SIGLEC9, APOBEC3C, HLA-DRB1, IFNGR2, ILF3, ISG20, ARF1, LCP1, LY75, MMP9, CD200, MYD88, ZBTB7B, PIK3CG, SASH3, PPP2R3C, DDX60, TRIM27, ACTB, SEMA4A, BMX, SP100, LAT2, IL1R2, TRIM11, HIST2H2BE, CASP8, NLRC5, PIAS1, IRS2, IL18R1, BCL10, KLHL6, ATG12, LPXN, TNFSF8</i>

KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.