Analysis of Peripheral Blood Cells' Transcriptome in Patients With Subarachnoid Hemorrhage From Ruptured Aneurysm Reveals Potential Biomarkers

Hua Zhao, Shi-ting Li, Jin Zhu, Xu-Ming Hua, Liang Wan

BACKGROUND: **Subarachnoid hemorrhage (SAH) is an uncommon disease. Considering ruptured intracranial aneurysms as the main cause of this disease and only a minority of the intracranial aneurysms will rupture sooner or later, to understand the underlying pathology or a specific gene expression profile of an impending ruptured intracranial aneurysm is of great importance.**

METHODS: **The transcriptome in peripheral blood cells of patients with SAH from ruptured aneurysm was compared with that of control patients suffering from headaches. The microarray dataset GSE36791 comprised 43 patients with SAH from ruptured aneurysms and 18 control patients. Differential expression analysis was performed with the R language packages to identify differentially expressed genes (DEGs). Gene Ontology enrichment analysis and Kyoto Encyclopedia of Genes and Genomes pathway database analysis were performed to identify significantly altered biological functions and pathways, respectively. The protein-protein interaction networks were constructed with information from the STRING database.**

RESULTS: **A total of 528 DEGs were identified, of which 311 were upregulated and 217 downregulated. Clustering analysis confirmed that these genes can distinguish ruptured aneurysm SAH patients from the control patients. The DEGs were mainly enriched for immune/inflammation response and related pathways. Among the DEGs, 8 genes (ARG1, MAPK14, RPS2, SPI1, FYN, CEBPB, FLOT1, and CD4) were identified as the key regulators in the Protein-Protein Interaction network. MAPK14, CEBPB, FLOT1, and CD4 might be potential biomarkers of SAH.**

CONCLUSION: **This study identified a range of DEGs SAH patients with ruptured aneurysms, which may enhance our current knowledge on this disease and may provide potential biomarkers of this disease.**

Key words
- Gene expression omnibus
- Peripheral blood cells' transcriptome
- Subarachnoid hemorrhage

Abbreviations and Acronyms
DEG: Differentially expressed gene
GO: Gene ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
PPI: Protein-protein interaction
SAH: Subarachnoid hemorrhage

INTRODUCTION

Subarachnoid hemorrhage (SAH) is an uncommon disease affecting patients at a mean age of 55 years with an incidence of about 9.1 cases per 100,000 people per year, which accounts for 5% of strokes, but is higher in Japan (22.7 cases per 100,000 people per year) and Finland (19.7 cases per 100,000 people per year).[1-3] The rupture of an intracranial aneurysm is the main cause of this disease in 85% of cases.[1] Aneurysmal SAH is a severe disease.[4] Modern 30-day mortality is as high as 40%, and about 50% of survivors have permanent disabilities.[5] It is difficult for emergency physicians to diagnose SAH. In the present study, after downloading an existing microarray data set (GSE36791) from the Gene Expression Omnibus databases, we performed a comparative analysis of peripheral blood cells' gene expression data between patients with SAH from ruptured aneurysm and patients suffering from headaches to identify the differentially expressed genes (DEGs), which may allow us to provide potential biomarkers for this disease.

MATERIALS AND METHODS

Gene Expression Data Set and Pretreatment

The microarray data set, GSE36791, was downloaded from the Gene Expression Omnibus databases (http://www.ncbi.nlm.nih.gov/gds/). This data set was provided by Professor J. Pera, who used the Illumina HumanHT-12 V4.0 expression bead chip for the analysis. The data comprised peripheral blood cell samples of 43 patients with SAH from ruptured aneurysm and 18 patients (used as control subjects) suffering from headaches.

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Table 1. The Genes that Were Upregulated by a Fold-Change >1.5 and With a *P* Value <0.05 (Top 30)

Gene Symbol	Entrez Gene ID	Fold Change
C19ORF59	199675	3.93322543
CA1	759	3.716513072
IL1R2	7850	3.282933409
ARG1	383	3.252301145
ANXA3	306	3.043297171
IL18RAP	8807	2.808810802
HP	3240	2.783807898
IL18R1	8809	2.781015933
MMP9	4318	2.688580727
ANKRD22	118932	2.669763601
OLFM4	10562	2.66553075
CST7	8530	2.624660948
ZDHHC19	131540	2.619735835
FOLR3	2352	2.601023417
GYG1	2992	2.555116978
IRAK3	11213	2.474592151
C5ORF32	84418	2.447685672
TNFAIP6	7130	2.420153193
DEFA4	1669	2.409129383
SLC2A11	66035	2.401121138
S100A12	6283	2.390636973
CD177	57126	2.348359949
PFKFB3	5209	2.34077293
BPGM	669	2.329439635
MOSC1	64757	2.260457324
OLAH	55301	2.259914237
LCN2	3934	2.240182142
GYPB	2994	2.238430028
ELANE	1991	2.217063572
CDK5RAP2	55755	2.207623391

Table 2. The Genes that Were Downregulated by a Fold-Change <0.6667 and With a *P* Value <0.05 (Top 30)

Gene Symbol	Entrez Gene ID	*P* Value	Fold Change
FCER1A	2205	1.21E-06	0.361519412
CD27	939	4.88E-07	0.463806404
CCR7	1236	1.00E-05	0.468625932
LEF1	51176	2.31E-06	0.482207835
BCL11B	64919	9.55E-08	0.496347601
IL2RB	3560	1.26E-08	0.497708563
CD6	923	1.28E-08	0.512240047
ABLIM1	3983	6.02E-07	0.512788896
CD7	924	4.92E-07	0.515953808
MAL	4118	1.93E-05	0.518653052
FLT3LG	2323	9.49E-07	0.522334362
EVL	51466	2.50E-07	0.522813958
ETS1	2113	0.000307	0.523466076
CLC	1178	0.002693	0.524360928
HDC	3067	0.000219	0.526063975
SPOCK2	9806	8.28E-09	0.526825741
FAIM3	9214	1.94E-07	0.536059968
CD247	919	3.31E-07	0.537846799
RPS4X	6191	1.32E-08	0.538096737
TNFRSF25	8718	1.31E-07	0.543393485
CD3D	915	1.36E-07	0.547324292
CD96	10225	1.36E-07	0.55085883
LDHB	3945	2.48E-06	0.552228983
SCGB3A1	92304	0.007427	0.554013853
KLRB1	3820	1.32E-06	0.555668435
FCGBP	8857	9.74E-06	0.556182968
ITK	3702	1.05E-07	0.556923121
GZMK	3003	5.96E-07	0.558061144
CD3E	916	3.77E-07	0.559138863
GNLY	10578	0.000295	0.565181423

After being background corrected, raw data was transformed with a log2 method and probes mapped to genes according to the annotation file; raw probe intensities were normalized with the Robust Multichip Average,[6] the average expression value for each gene was calculated for all probes corresponding to the same gene.

Identification of DEGs

The DEGs between the 2 groups were screened using the R language packages.[7] In detail, the fold-change (linear) ≤0.6667 or fold change (linear) ≥1.5, and a paired t test P value <0.05 were used as the cutoffs. Cluster software[8] and TreeView software[9] were used to perform hierarchical clustering of the DEGs. Also, scatterplots were drawn to visually observe the DEGs.

Functional Enrichment Analysis and Related Signaling Pathways Analysis for DEGs

For Gene Ontology (GO) enrichment analysis (including cell components, molecular functions, and biological processes), the list of DEGs was uploaded to the DAVID website (http://david.abcc.ncifcrf.gov/)[10] and screened with both Fisher's exact test P value and false discovery rate <0.05. For related signaling pathways

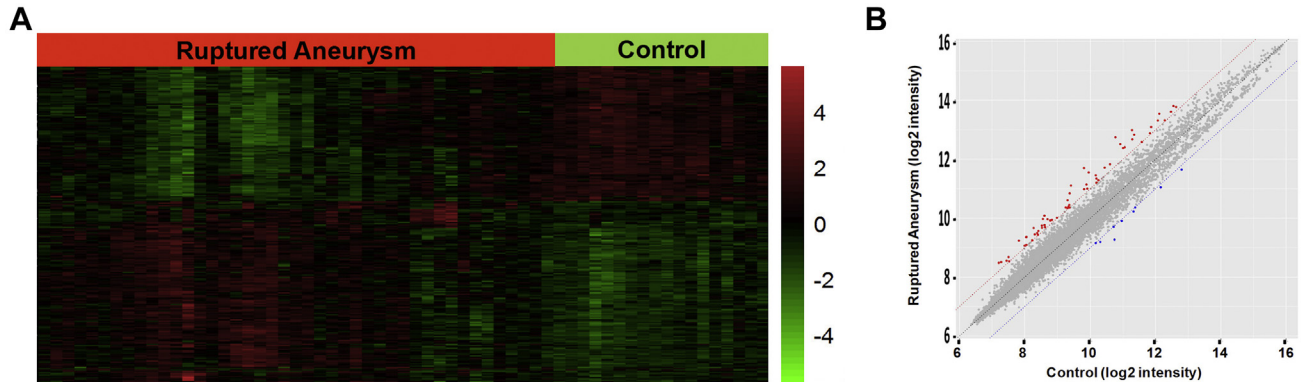

Figure 1. Clustering analysis and scatter plot of differentially expressed genes. (**A**) Clustering analysis of differentially expressed genes between ruptured aneurysm and control subjects. Data were derived from the log2 transformation for each gene. Red, high expression; green, low expression, according to Z scores. (**B**) Global overview of differential gene expression determined by microarray analysis comparing ruptured aneurysm with control subjects in scatter plot.

analysis for DEGs, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[11] was used, and Fisher's exact test *P* value <0.05 was set as the cutoff.

Construction of a Protein-Protein Interaction Network

Those genes in the DEGs enriched in the top 20 GO functional enrichment or top 20 KEGG pathways were used to obtain the protein-protein interactions (PPI) with score >0.7 using the STRING database (http://string-db.org/). Then, Cytoscape 3.0 software[12] was used to construct a PPI network with the obtained interactions in STRING database, thus identifying the key genes in the network.

Gene Set Enrichment Analysis

To explore the associated pathways of the 8 key genes, we first identified the coexpressed genes (Pearson correlation analysis, $P < 0.05$) with each of the 8 genes we analyzed. Then Gene Set Enrichment Analysis was performed between the positively and negatively correlated coexpressed genes of each of the 8 genes to reveal their associated pathways.

RESULTS

DEGs

A total of 528 DEGs were identified comparing between samples of patients with SAH from ruptured aneurysm (from the ruptured aneurysm group) and of patients suffering from headaches (the control group) at paired t test *P* value <0.05 and fold change (linear) ≤0.6667 or ≥1.5, including 311 upregulated genes (**Table S1**; **Table 1**) and 217 downregulated genes (**Table S1**; **Table 2**). The clustering analysis result and scatter plot are shown in **Figure 1**, indicating these genes may be used to distinguish between the ruptured aneurysm group and the control group.

GO Enrichment Analysis for DEGs

The GO enrichment analysis showed that the DEGs were mainly enriched for GO terms such as "translation," "T cell activation," "innate immune response," "immunoglobulin mediated immune response," indicating that these genes are mainly associated with immune response (**Figure 2A**). The top 20 GO terms enriched by the DEGs are shown in **Figure 2A**, and the detailed results of GO analysis are shown in **Table S2**.

KEGG Pathway Analysis for DEGs

The KEGG pathways analysis showed that the DEGs were mainly enriched for the KEGG pathway terms such as "ribosome," "hematopoietic cell lineage," "transcriptional misregulation in cancer," "T cell receptor signaling pathway," also indicating these genes are closely associated with immune function and hematopoietic function (**Figure 2B**). The top 20 KEGG pathway terms enriched by the DEGs are shown in **Figure 2B**, and the detailed results of KEGG pathway analysis are shown in **Table S3**.

PPI Network

Genes among the DEGs that were either enriched in the top 20 GO functional enrichment or top 20 KEGG pathways were included to construct a PPI network using the STRING online tools and Cytoscape 3.0 software. Finally, a total of 104 genes were included in the PPI network, including 51 upregulated genes and 53 downregulated genes (**Figure 3**; **Table S4**). In this network, the Betweenness Centrality value and average degree of each node gene was analyzed, and the genes with a high Betweenness Centrality value were considered to be "hub" genes. To this end, we identified ARG1, MAPK14, RPS2, SPI1, FYN, CEBPB, FLOT1, and CD4 as the hub genes in this study (**Figure 3**; **Table 3**).

Gene Set Enrichment Analysis

We found that ARG1, SPI1, and CD4 were upregulated and that the other 5 genes were downregulated in unruptured aneurysm in comparison with the normal control. Thus, MAPK14, CEBPB, and FLOT1 were specifically upregulated and that CD4 were specifically downregulated in the pathologic process from unruptured to ruptured aneurysm (**Figure 4**).

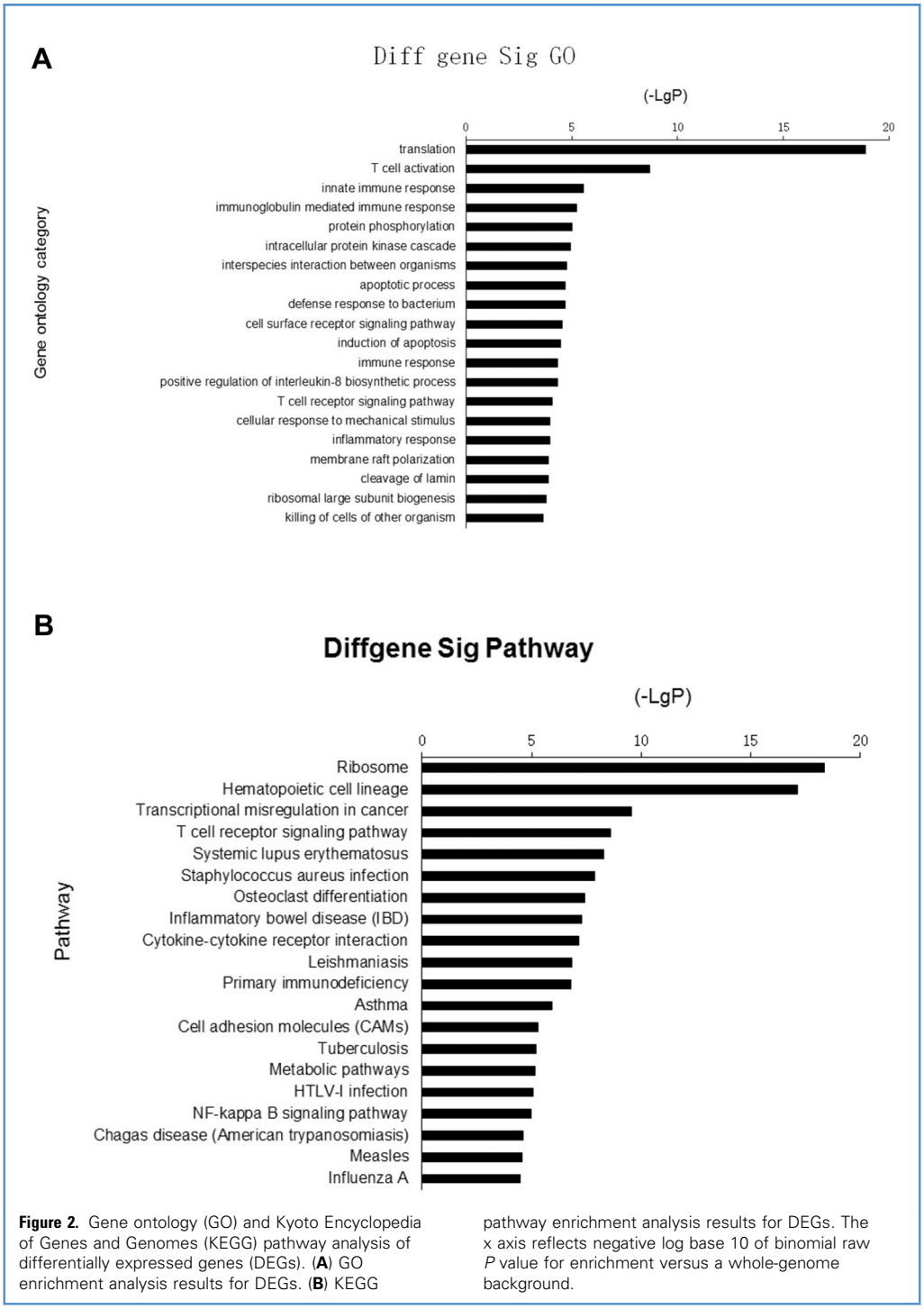

Figure 2. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes (DEGs). (**A**) GO enrichment analysis results for DEGs. (**B**) KEGG pathway enrichment analysis results for DEGs. The x axis reflects negative log base 10 of binomial raw *P* value for enrichment versus a whole-genome background.

DISCUSSION

The most common symptom of aneurysmal SAH is abrupt strong headache[1]; however, headache is a nonspecific feature of aneurysmal SAH because in general practice most headaches are innocuous. This causes much difficulty to diagnose SAH for emergency physicians. The diagnosis can easily be missed considering the relatively low incidence, but once misdiagnosed and missing the best timing of treatment, most patients will have increased risk of death and severe disability.[13] Nowadays, the application of multidetector computed tomography scanners

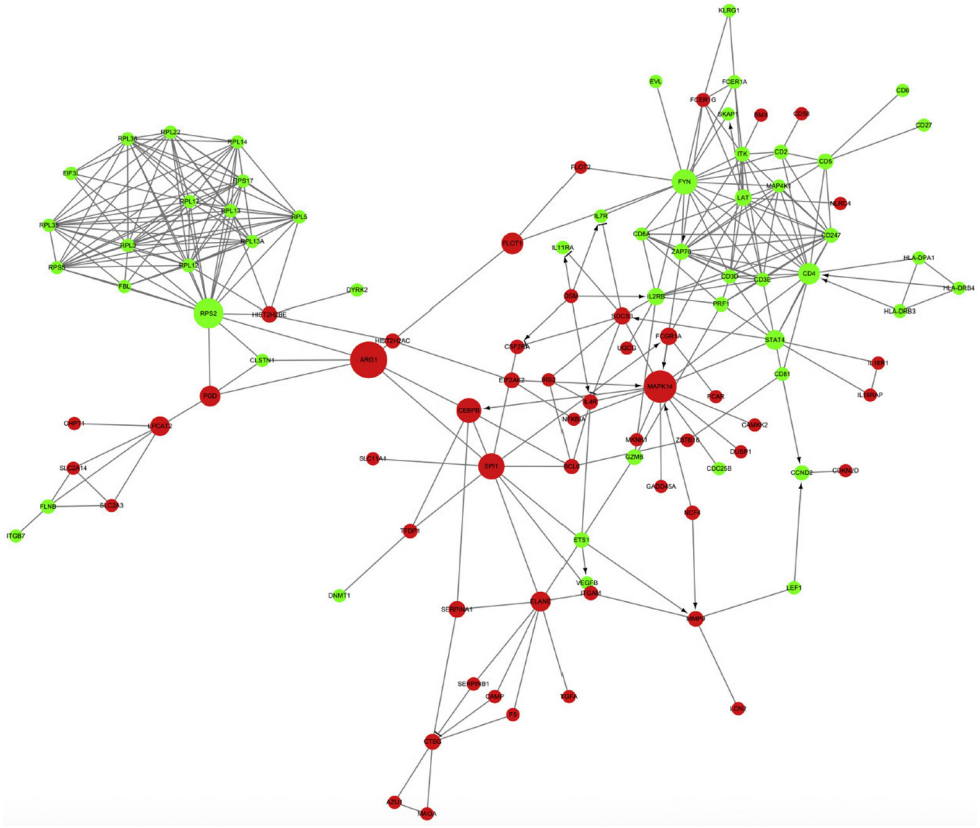

Figure 3. The protein-protein interaction network of selected differentially expressed gene products. Upregulated proteins in the ruptured aneurysm group are in red (n = 51), downregulated proteins are in green (n = 54).

can obtain a high sensitivity of diagnosing SAH, but mainly within the first 6 hours and declined with increasing time from headache onset.[13,14] Also, for those aneurysms that present with SAH, there is still high risk of rerupture, especially within the first 2 weeks of the initial event.[15] In addition, sooner or later, a portion of unruptured aneurysms may rupture in the future, and this segment of patients should be treated as soon as possible.[16] However, there has not been an efficient method to precisely predict rupture of an unruptured aneurysm or rerupture of aneurysmal SAH until now, even taking multidetector computed tomography scanners into account. Therefore, new methods should be found to solve this problem.

Table 3. Hub Genes Identified in the PPI Network

Gene Name	Betweenness Centrality	Degree	Fold Change
ARG1	0.351356	6	Up
MAPK14	0.286284	15	Up
RPS2	0.242122	17	Down
SPI1	0.191952	10	Up
FYN	0.177372	18	Down
CEBPB	0.172731	6	Up
FLOT1	0.125892	3	Up
CD4	0.124522	16	Down

Understanding the pathophysiology and molecular characteristics of this disease will enable novel diagnostics for improved patient selection in personalized medicine trials for SAH.[17] Recent advancements in high-throughput technology have enabled the identification of molecular biomarkers that may prove useful in diagnosing and managing patients with unruptured aneurysms or with aneurysmal SAH. It is known that there is a systemic response to SAH pathogenesis that includes pulmonary edema, heart contractility abnormalities, fluid and electrolyte balance, and systemic inflammatory response syndrome.[1,18] Common mechanisms for this systemic response are increased sympathetic nervous system activity, with increased catecholamine, natriuretic peptide, renin or angiotensin system activation, and inflammatory cytokines. We further hypothesized that special changes of expression profiles might also occur in peripheral blood cells during the systemic response to a rupture of intracranial

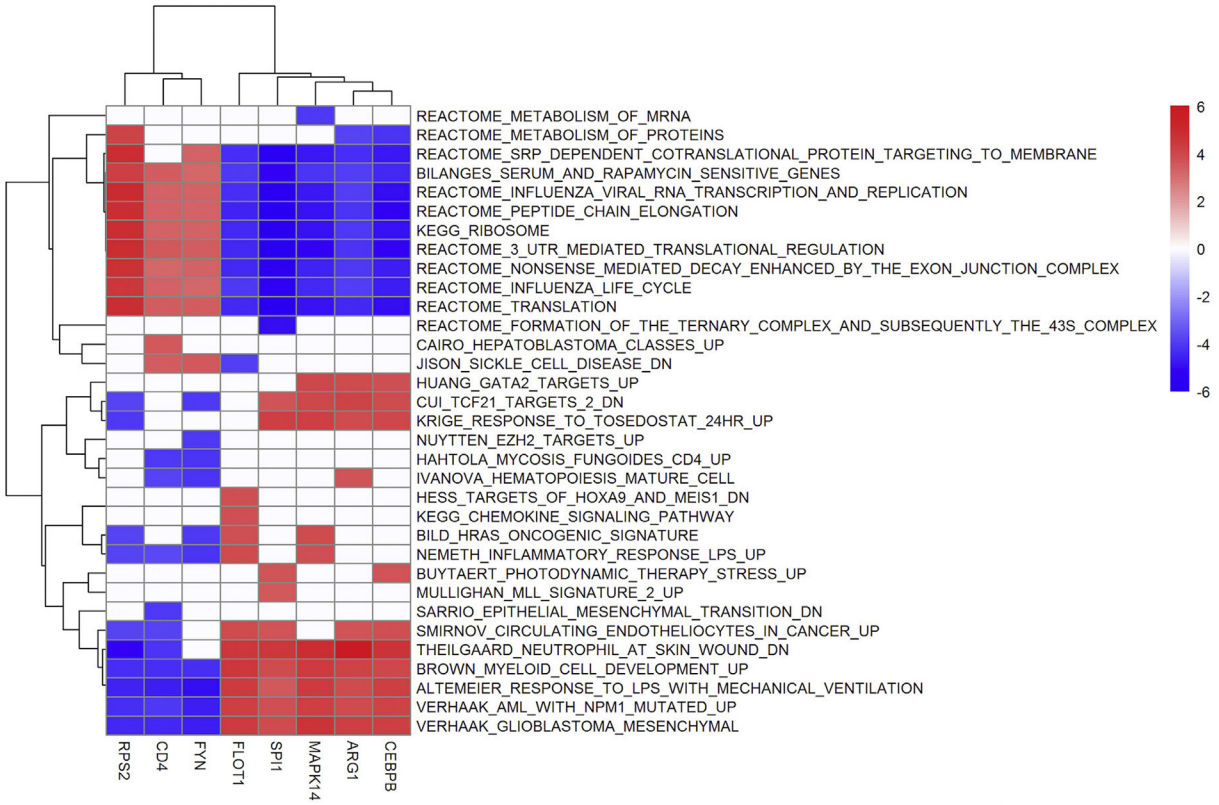

Figure 4. Gene set enrichment analysis was performed between the positively and negatively correlated coexpressed genes of each of the 8 genes.

aneurysms or in the clinical status of SAH patients, considering that testing potential biomarkers in peripheral blood cells might be a simple, economic, and efficient way.

In this study, by analyzing the transcriptome in peripheral blood cells, a total of 528 DEGs were identified in patients with SAH from ruptured aneurysm compared with control patients suffering from headaches, of which 311 were upregulated and 217 downregulated. GO enrichment analysis and KEGG pathway analysis revealed that these DEGs were significantly enriched for the immune/inflammatory system and relevant pathways. PPI network analysis revealed that 8 genes (ARG1, MAPK14, RPS2, SPI1, FYN, CEBPB, FLOT1, and CD4) among the DEGs were the key regulators in the network. To further confirm whether the 8 key genes are promising candidates as biomarkers of SAH or impending ruptured intracranial aneurysms, we analyzed another dataset, GSE26969, which includes 3 samples from patients with unruptured intracranial arterial aneurysm and 3 normal control samples. We found that ARG1, SPI1, and CD4 were upregulated and that the other 5 genes were downregulated in unruptured aneurysm in comparison with normal control. Thus, MAPK14, CEBPB, and FLOT1 were specifically upregulated and that CD4 were specifically downregulated in the pathologic process from unruptured to ruptured aneurysm. MAPK14, CEBPB, FLOT1, and CD4 might be potential biomarkers of SAH.

SAH is a devastating and complicated disease with poor prognosis for most patients.[19] Considering that ruptured intracranial aneurysms are the main cause of this disease and only a minority of the intracranial aneurysms will rupture, to understand the underlying pathology or a specific gene expression profile of an impending ruptured intracranial aneurysm is of great importance. Lee et al.[20] found that the gene expression profiles between ruptured and unruptured (but with a high risk to rupture) human intracranial aneurysms are similar, but different from that of normal intracranial arteries, indicating that the pathology status in a patient with a ruptured intracranial aneurysm may mirror the pathology status in a patient with an impending ruptured intracranial aneurysm, at least partly. Therefore, to a considerable degree, factors leading to the pathological findings made in ruptured aneurysms may already exist before rupture occurs.[21] In our present study, we obtained several unique genes that were differentially expressed in ruptured aneurysm SAH patients compared with control patients suffered from headaches. We think these genes may act as biomarkers of SAH from ruptured aneurysm or an impending ruptured aneurysm.

Immune response is closely related to SAH, and there is a lot of evidence that inflammatory cascade plays an important role in SAH. The immune cells in the blood are always closely related to

this disease. For example, macrophage infiltrates, leukocyte inflammation and increased levels of the inflammatory mediators have been frequently found in the walls of ruptured aneurysms.[22-25] Most of these results were found in human aneurysm tissue samples; however, human aneurysm tissue samples are always difficult to obtain. In fact, the most readily available samples are peripheral blood and cerebrospinal fluid. Therefore, here, in this study, we performed a comparative analysis of peripheral blood cells' gene expression data between patients with SAH from ruptured aneurysms and patients suffering from headaches and found the DEGs were significantly enriched for the immune/inflammatory response and relevant pathways. Our results indicate that the gene expression profiles in peripheral blood cells can reflect the status of the disease, thus acting as potential easy-to-detect biomarkers of this disease.

CONCLUSION

In summary, the present study describes the differential expression profiles in peripheral blood cells between patients with SAH from ruptured aneurysms and patients suffering from headaches. The identified DEGs, especially the 4 key genes (MAPK14, CEBPB, FLOT1, and CD4), are promising candidates' biomarkers of SAH or impending ruptured intracranial aneurysms. Our results should also be further validated in a larger sample size of patients.

REFERENCES

1. Macdonald RL, Schweizer TA. Spontaneous subarachnoid haemorrhage. *Lancet*. 2017;389:655-666.
2. Feigin VL, Lawes CM, Bennett DA, Barker-Collo SL, Parag V. Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. *Lancet Neurol*. 2009;8:355-369.
3. de Rooij NK, Linn FH, van der Plas JA, Algra A, Rinkel GJ. Incidence of subarachnoid haemorrhage: a systematic review with emphasis on region, age, gender and time trends. *J Neurol Neurosurg Psychiatry*. 2007;78:1365-1372.
4. Abraham MK, Chang WW. Subarachnoid hemorrhage. *Emerg Med Clin North Am*. 2016;34:901-916.
5. Dority JS, Oldham JS. Subarachnoid hemorrhage: an update. *Anesthesiol Clin*. 2016;34:577-600.
6. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4:249-264.
7. Dessau RB. Pipper CB ["R"–project for statistical computing.]. *Ugeskr Laeger*. 2008;170:328-330.
8. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A*. 1998;95:14863-14868.
9. Page RD. TreeView: an application to display phylogenetic trees on personal computers. *Compt Appl Biosci*. 1996;12:357-358.
10. Huang DW, Sherman BT, Tan Q, et al. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol*. 2007;8:R183.
11. Kanehisa M. The KEGG database. *Novartis Found Symp*. 2002;247:91-101. discussion 101-103, 119-128, 244-152.
12. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13:2498-2504.
13. Kowalski RG, Claassen J, Kreiter KT, et al. Initial misdiagnosis and outcome after subarachnoid hemorrhage. *JAMA*. 2004;291:866-869.
14. Perry JJ, Stiell IG, Sivlotti ML, et al. Sensitivity of computed tomography performed within six hours of onset of headache for diagnosis of subarachnoid haemorrhage: prospective cohort study. *BMJ*. 2011;343:d4277.
15. Brisman JL, Song JK, Newell DW. Cerebral aneurysms. *N Engl J Med*. 2006;355:928-939.
16. Wiebers DO, Whisnant JP, Sundt TM Jr, O'Fallon WM. The significance of unruptured intracranial saccular aneurysms. *J Neurosurg*. 1987;66:23-29.
17. Achrol AS, Steinberg GK. Personalized medicine in cerebrovascular neurosurgery: precision neurosurgical management of cerebral aneurysms and subarachnoid hemorrhage. *Front Surg*. 2016;3:34.
18. Chen S, Li Q, Wu H, et al. The harmful effects of subarachnoid hemorrhage on extracerebral organs. *Biomed Res Int*. 2014;2014:858496.
19. Provencio JJ, Vora N. Subarachnoid hemorrhage and inflammation: bench to bedside and back. *Semin Neurol*. 2005;25:435-444.
20. Lee CI, Chou AK, Lin CC, et al. Immune and inflammatory gene signature in rat cerebrum in subarachnoid hemorrhage with microarray analysis. *Mol Med Rep*. 2012;5:118-125.
21. Pera J, Korostynski M, Krzyszkowski T, et al. Gene expression profiles in human ruptured and unruptured intracranial aneurysms: what is the role of inflammation? *Stroke*. 2010;41:224-231.
22. Kataoka K, Taneda M, Asai T, Kinoshita A, Ito M, Kuroda R. Structural fragility and inflammatory response of ruptured cerebral aneurysms. A comparative study between ruptured and unruptured cerebral aneurysms. *Stroke*. 1999;30:1396-1401.
23. Chalouhi N, Ali MS, Jabbour PM, et al. Biology of intracranial aneurysms: role of inflammation. *J Cereb Blood Flow Metab*. 2012;32:1659-1676.
24. Lanzino G. Inflammation after embolization of intracranial aneurysms. *J Neurosurg*. 2008;108:1071-1073.
25. Chyatte D, Bruno G, Desai S, Todor DR. Inflammation and intracranial aneurysms. *Neurosurgery*. 1999;45:1137-1146.

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