Identification of potential gene targets in systemic vasculitis using DNA microarray analysis

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Received December 21, 2015; Accepted January 27, 2017

DOI: 10.3892/mmr.2017.6455

Abstract. The present study aimed to identify the involvement of critical genes in systemic vasculitis, to gain an improved understanding of the molecular circuity and to investigate novel potential gene targets for systemic vasculitis treatment. The dual-color cDNA microarray data of GSE16945, consisting of peripheral mononuclear blood cell specimens from 13 patients with systemic vasculitis and 16 healthy controls, was downloaded from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were screened in systemic vasculitis compared with controls using BRB ArrayTools, followed by the construction of a protein-protein interaction (PPI) network using the clusterProfiler package, and significant functional interaction (FI) module selection. Furthermore, transcriptional factors (TFs) among the identified DEGs were predicted and a transcriptional regulation network was constructed. A total of 173 up- and 93 downregulated genes were identified, which were mainly associated with immune response pathways. FBJ murine osteosarcoma viral oncogene homolog (FOS), ubiquitin B (UBB), signal transducer and activator of transcription 1 (STAT1) and MX dynamin-like GTPase 1 (MX1) were identified as hub proteins in the PPI network. Furthermore, UBB, FOS, and STAT1 were hub proteins in the three identified FI modules, respectively. In total, nine TFs were predicted among the DEGs. Of the DEGs that were predicted to be TFs, STAT1, v-maf avian musculosarcoma fibrosarcoma oncogene homolog B (MAFB) and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein Z (YWHAZ), which interacted with each other, were identified to regulate further DEGs as target genes. Various genes, including FOS, UBB, MX1, STAT1, MAFB, and YWHAZ may be potential targets useful for the treatment of systemic vasculitis.

Introduction

Systemic vasculitis represents a heterogeneous group of disorders characterized by inflammation and necrosis in the blood vessel wall involving multiple organs (1). Clinical manifestations of systemic vasculitis are nonspecific and may vary from mild disorders to life-threatening multisystem conditions (2). Cases of vasculitis exhibit differences in pathology, type of inflammation, vessel and organ involvement, and demographics (3). Therefore, the diagnosis and treatment of systemic vasculitis is challenging, and improvements in therapy and the understanding of the etiopathogenesis are required. Although the etiology of systemic vasculitis is not fully understood, there are a combination of genetic, immunological and environmental factors that may be responsible for certain cases. Accumulating evidence has demonstrated that genetic factors contribute to the susceptibility to vasculitis (4). Anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV) is a necrotizing group of disorders characterized by autoimmune inflammation that predominantly affects small to medium-sized vessels, which can lead to vessel occlusion and systemic organ damage (5). Ordonez et al (6) reported that CD45RC⁺ CD4 T cells were significantly increased in patients with AAV compared with healthy controls, which may contribute to the susceptibility to AAV. Additionally, Kobayashi et al (7) demonstrated that putative gene markers, particularly early growth response 1 and G0/G1 switch gene 2, may be useful for diagnosing vasculitis, and monocytes expressing these vasculitis-upregulated genes may be involved in the pathogenesis of vasculitis. However, the molecular mechanism underlying the development and progression of systemic vasculitis remains unclear. Continued investigation and identification of the genetic factors involved in the pathogenesis of systemic vasculitis is necessary.

Dual-color cDNA microarray data was used in the present study to identify differentially expressed genes (DEGs) in samples from patients with systemic vasculitis compared with healthy controls. Comprehensive bioinformatics were
conducted to analyze the significant gene ontology (GO) terms and pathways that the DEGs were involved in. This was followed by the construction of a protein-protein interaction (PPI) network and significant functional interaction (FI) module selection. Furthermore, transcriptional factors (TFs) among the DEGs were predicted and, subsequently, a transcriptional regulation network was constructed. The current study aimed to identify the involvement of critical genes in systemic vasculitis, to obtain an improved understanding of the molecular circuitry in systemic vasculitis and to investigate novel potential gene targets for systemic vasculitis treatment.

Materials and methods

Data source. The dual-color cDNA microarray data GSE16945 (8), was downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) based on the platform of GPL4133 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F (feature number version). This dual-color microarray dataset consisted of details of 13 microarray chips. Each chip included a cyanine (Cy) 3-labelled channel derived from mixed peripheral mononuclear blood cells (PMBCs) from 16 healthy volunteers as controls and a Cy5-labelled channel derived from PMBCs from a patient with systemic vasculitis. A total of 13 patients with vasculitis were included in the present study.

Data preprocessing and DEG screening. Raw data were imported into BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html; version 4.3.1) developed by Simon et al (9). The preprocessing step included local background subtraction, averaging of intensities of duplicated probes and quantile normalization across multiple arrays. DEG screening was performed via the BRB ArrayTools package (9). Genes were excluded when <50% of the expression data had >1.5-fold change in either direction from the median value of the gene. Genes were also excluded when the percentage of data filtered out or missing was >50%. The threshold for a DEG was P<0.05 using multivariate permutation tests as previously described (10).

Functional and pathway enrichment analysis. The clusterProfiler package is implemented in R, which is an open-source programming environment (11) and this package automates the process of biological-term enrichment analysis of gene clusters (12). In the present study, the clusterProfiler package version 3.4 (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) was applied to perform GO analysis (including cellular composition, molecular function and biological process terms) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. False discovery rate (FDR) (13) was performed to adjust P-values using the Benjamini and Hochberg method (14). An FDR <0.05 was selected as the cutoff criterion.

PPI network construction. A PPI network was constructed in the present study using the Search Tool for the Retrieval of Interacting Genes database (15). Interacting pairs of DEGs (confidence score >0.4) (16) were selected for PPI network construction. Additionally, Cytoscape software version 2.8 (17) was used to provide interactive visualization for the PPI network. The node degrees in the PPI network were calculated with the igraph package version 0.5.3 (18) in R and the nodes with higher degrees were considered to be hub proteins.

FI network construction and modules selection. ReactomeFIVis (19), a Cytoscape plugin, integrates constructed human protein FI networks by combining curated and non-curated data sources, and the gene expression matrix, to calculate the Pearson coefficient of genes (20) in an FI network. In the FI network, the Pearson coefficient of each pair of genes were considered as edge weights. In addition, ReactomeFIVis further divides the FI network into modules using a popular algorithm, Markov Clustering (MCL) (21). In the current study, a gene FI network was constructed using ReactomeFIVis (19) and module division was performed. Furthermore, Gene Cluster with Literature Profiles (GenCLiP) software version 2.0, which was developed by Huang et al (22) to cluster gene lists by literature profiling and to construct gene co-occurrence networks associated with custom keywords, was used to analyze the biological behavior of genes identified in the selected modules.

Prediction of TFs and transcriptional regulation network construction. TRANSFAC is a database on TFs, which contains the genomic binding sites of TFs and DNA-binding profiles, and is an integrated system for gene expression regulation (23). TFs among the identified DEGs were screened for using data derived from TRANSFAC database in the present study. Candidate binding sites for the identified TFs in the promoter region were also identified through sequence matching of the position weight matrix (24) by using the MotifDb R package (25). The location of the TF binding site (TFBS) within the promoter region of each DEG was predicted using the position weight matrix algorithm, in which a minimum score for a match was set at 85%. Subsequently, a transcriptional regulation network was constructed that included the identified TFs and other DEGs.

Results

Screening of DEGs. A total of 266 DEGs were identified in PMBCs from patients with systemic vasculitis compared with controls, including 173 up- and 93 downregulated genes. The results demonstrated that there were more upregulated genes than downregulated genes in patients with vasculitis compared with controls.

GO and pathway enrichment analysis. GO and pathway analysis demonstrated that up- and downregulated genes were associated with different GO terms and pathways. The top five GO terms in each category of the up- and downregulated genes are presented in Table I. Additionally, the top five KEGG pathways associated with the up- and downregulated genes are presented in Table II. The results of the present study demonstrated that upregulated DEGs were primarily involved in biological processes associated with defense response and response to stress or stimulus. However, downregulated DEGs were enriched in biological processes associated with immune responses, such as the innate immune response.
PPI network construction and analysis. The PPI network was constructed and is presented in Fig. 1. The PPI network included 163 nodes and 449 interactions, involving 107 up- and 56 downregulated genes. Following the calculation of node degrees, the present study identified that the top five DEGs in the PPI network, with higher node degrees, were FBJ murine osteosarcoma viral oncogene homolog (FOS; degree, 23), ubiquitin B (UBB; degree, 22), ISG15 ubiquitin-like modifier (degree, 22), signal transducer and activator of transcription 1 (STAT1; degree, 21) and MX dynamin-like GTPase 1 (MX1; degree, 17). Furthermore, a total of nine TFs that were involved in the PPI network were predicted, including cold shock domain protein A, Fas cell surface death receptor, high mobility group box 2, interferon regulatory factor 5, v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), myocyte enhancer factor 2D (MEF2D), POU class 2 associating factor 1, STAT1, tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein Z (YWHAZ).

Module selection in the FI network and function analysis of DEGs in the modules. The FI network of DEGs was

| GO ID            | Description                                      | P-value      | False discovery rate | Count |
|------------------|--------------------------------------------------|--------------|----------------------|-------|
| **Upregulated**  |                                                  |              |                      |       |
| GO_BP 0006950    | Response to stress                               | 2.35E-12     | 2.40E-09             | 65    |
| GO_BP 0002376    | Immune system process                            | 6.88E-12     | 2.90E-09             | 51    |
| GO_BP 0006952    | Defense response                                 | 8.52E-12     | 2.90E-09             | 40    |
| GO_BP 0006954    | Inflammatory response                            | 3.24E-10     | 8.28E-08             | 23    |
| GO_BP 0050896    | Response to stimulus                             | 9.57E-10     | 1.96E-07             | 101   |
| GO_CC 0031982    | Vesicle                                          | 7.96E-09     | 6.77E-07             | 58    |
| GO_CC 0044421    | Extracellular region part                         | 1.32E-08     | 6.77E-07             | 59    |
| GO_CC 005833     | Hemoglobin complex                               | 1.51E-08     | 6.77E-07             | 5     |
| GO_CC 005615     | Extracellular space                              | 3.96E-08     | 1.33E-06             | 30    |
| GO_CC 0031988    | Membrane-bounded vesicle                         | 1.69E-07     | 3.80E-06             | 54    |
| GO_MF 0003674    | Molecular function                               | 3.42E-10     | 4.82E-08             | 146   |
| GO_MF 0005515    | Protein binding                                  | 3.00E-08     | 1.33E-06             | 100   |
| GO_MF 0005488    | binding                                          | 3.11E-08     | 1.33E-06             | 129   |
| GO_MF 0005344    | Oxygen transporter activity                       | 3.76E-08     | 1.33E-06             | 5     |
| GO_MF 0005506    | Iron ion binding                                 | 8.50E-06     | 0.000212228          | 9     |
| **Downregulated**|                                                  |              |                      |       |
| GO_BP 0006952    | Defense response                                 | 2.30E-16     | 8.58E-14             | 34    |
| GO_BP 0006955    | Immune response                                  | 2.46E-16     | 8.58E-14             | 33    |
| GO_BP 0045087    | Innate immune response                           | 4.35E-14     | 1.01E-11             | 25    |
| GO_BP 0002376    | Immune system process                            | 7.59E-14     | 1.32E-11             | 38    |
| GO_BP 0051607    | Defense response to virus                        | 4.92E-13     | 6.86E-11             | 14    |
| GO_CC 0044444    | Cytoplasmic part                                 | 1.86E-06     | 0.000161279          | 55    |
| GO_CC 005737     | Cytoplasm                                        | 2.56E-06     | 0.000161279          | 66    |
| GO_CC 0042611    | MHC protein complex                              | 7.12E-06     | 0.000298999          | 4     |
| GO_CC 0071556    | Integral component of lumenal side of endoplasmic reticulum membrane | 0.000294636 | 0.005946701 | 3     |
| GO_CC 0098553    | Lumenal side of endoplasmic reticulum membrane   | 0.000294636 | 0.005946701 | 3     |
| GO_MF 0003674    | Molecular function                               | 3.66E-06     | 0.00019454           | 84    |
| GO_MF 0003823    | Antigen binding                                  | 4.42E-06     | 0.00019454           | 6     |
| GO_MF 0005515    | Protein binding                                  | 1.59E-05     | 0.000466985          | 58    |
| GO_MF 0003924    | GTPase activity                                  | 6.22E-05     | 0.001368961          | 7     |
| GO_MF 0005525    | GTP binding                                      | 0.000282526 | 0.003941692 | 8     |

GO, gene ontology; BP, biological process; CC, cellular composition; MF, molecular function. Count, the number of DEGs that this specific GO-ID term was associated with.
constructed using the ReactomeFIViz plugin. Furthermore, three significant modules were identified using the MCL algorithm (Fig. 2). *UBB*, *FOS* and *STAT1* were identified as hub proteins for one of the three identified modules. In addition, these three DEGs interacted with each other, and *FOS* and *STAT1* were identified as TFs following literature profiling. As presented in Fig. 2, there were seven, nine and seven DEGs enriched in modules 1, 2 and 3, respectively. Additionally, according to the clustered gene lists by literature profiling, it was identified that the upregulated DEG, *UBB*, which was the hub protein in module 1, was associated with cell death and signal transduction (Fig. 3). While the hub protein in module 2, *FOS* (upregulated), as an important TF, was associated with inflammatory response, signal transduction and cell

Table II. Enriched KEGG pathways for up- and downregulated genes in patients with systemic vasculitis.

| KEGG ID   | Description                                      | P-value       | False discovery rate | Count |
|-----------|--------------------------------------------------|---------------|----------------------|-------|
| hsa05130  | Pathogenic *Escherichia coli* infection          | 0.000614223   | 0.013512902          | 5     |
| hsa03010  | Ribosome                                         | 0.004807379   | 0.035254116          | 5     |
| hsa05323  | Rheumatoid arthritis                             | 0.004807379   | 0.035254116          | 5     |
| hsa05143  | African trypanosomiasis                          | 0.008320256   | 0.038297864          | 3     |
| hsa05146  | Amoebiasis                                       | 0.008704060   | 0.038297864          | 5     |
| hsa05330  | Allograft rejection                              | 0.000139043   | 0.001547821          | 4     |
| hsa05332  | Graft-versus-host disease                        | 0.000204470   | 0.001547821          | 4     |
| hsa04940  | Type I diabetes mellitus                         | 0.000244393   | 0.001547821          | 4     |
| hsa05320  | Autoimmune thyroid disease                       | 0.000495821   | 0.002355147          | 4     |
| hsa04145  | Phagosome                                        | 0.000652198   | 0.002478353          | 6     |

Count, the number of DEGs that the specific KEGG-ID was associated with.

Figure 1. Protein-protein interaction network of differentially expressed genes in patients with systemic vasculitis compared with controls. Red nodes indicate upregulated genes. Green nodes indicate downregulated genes. Diamond-shaped nodes indicate transcription factors.
Downregulated STAT1, which was the hub protein in module 3, was identified to be a TF that was associated with the immune response, inflammatory response, cell death and Toll-like receptors (TLRs).

**Construction and analysis of the transcriptional regulation network.** As mentioned above, a total of nine DEGs were predicted to be TFs based on data derived from the TRANSFAC database. The present study subsequently predicted all of the DEGs to be TFs.
potential target genes, which were also identified as DEGs, of these nine TFs. The results demonstrated that five TFs potentially regulated 257 DEGs (167 up- and 90 downregulated genes). In particular, it was identified that STAT1, MEF2D, MAFB, and YWHAZ potentially regulated further DEGs (Fig. 4). TFBS were also identified in pairs of TFs. The interactions among the nine identified TFs are presented in Fig. 5. Furthermore, genes that were coregulated by STAT1, MEF2D, MAFB, and YWHAZ were analyzed. The results indicated that TFs STAT1 and MAFB may coregulate 39 target genes, while MEF2D and YWHAZ may coregulate 70 target genes. These target genes were enriched in biological processes associated with response to environmental stimulus and immune response (Fig. 6).

Discussion

Gene expression profiling using DNA microarrays is a tool for investigating systemic vasculitis at a molecular level (4,7). In the current study, a total of 173 up- and 93 downregulated genes were identified in PMBCs from patients with systemic vasculitis compared with controls. GO and pathway enrichment analysis demonstrated that DEGs were primarily
associated with immune response. *FOS*, *UBB*, *STAT1* and *MX1* were identified as hub proteins in the PPI network. Furthermore, *UBB*, *FOS* and *STAT1* were hub proteins in one of the three identified FI modules. A total of nine TFs were predicted among the identified DEGs. Of those nine TFs, *STAT1*, *MAFB* and *YWHAZ*, which exhibited interactions among each other, were indicated to regulate further DEGs as target genes in the regulation network. The target genes of the TFs were primarily associated with response to environmental stimulus and immune response.

The *FOS* gene family encodes leucine zipper proteins, which dimerize with proteins of the *JUN* family, forming the TF complex activator protein-1 (AP-1) to regulate gene expression (5). Previous studies have demonstrated that the signaling of TLRs, which results in cytokine production, is integrated by adapter molecules that activate AP-1 in lipopolysaccharide-stimulated lung injury (26-28). In addition, Tadema *et al* (29) demonstrated that monocytes and natural killer cells exhibited increased TLR expression in AAV. Consistent with the previous study, the results of the current study revealed that *FOS* was upregulated in PMBCs from patients with systemic vasculitis compared with controls, and *FOS* was a hub protein in the PPI network and also in the FI module 2. In this context, we hypothesized that *FOS* may have a crucial role in the TLR signaling involved in the inflammatory response in systemic vasculitis, and, thus, *FOS* may be a potential gene target for vasculitis treatment.

*UBB* encodes ubiquitin, which is one of the most conserved proteins and has a major role in targeting cellular proteins for degradation by the 26S proteasome (30). It is apparent that ubiquitination of various components of the Notch signaling pathway functions in shaping and orchestrating the Notch signaling pathway (31). Additionally, Piggot *et al* (32) revealed that blocking the Notch pathway inhibited vascular inflammation in large-vessel vasculitis and modulating the Notch signaling cascade may be a promising novel method for immunosuppressive therapy of large-vessel vasculitis. Furthermore, the present study identified that *UBB* was upregulated in PMBCs from patients with systemic vasculitis compared with controls, and *UBB* was a hub protein in the PPI network and in the identified FI module 1. Combined, these results indicate that upregulated *UBB* may function in the progression of systemic vasculitis via participation in the Notch signaling pathway. Therefore, *UBB* may be a potential therapeutic target for systemic vasculitis, which requires further investigation.

The results of the present study also identified that *MX1* was another hub protein in the PPI network. *MX1* encodes a GTP-metabolizing protein, which is induced by type I and type II interferons and participates in the cellular antiviral response by antagonizing the replication process of several viruses (33). Evidence has demonstrated that the most common vasculitic syndrome associated with hepatitis C virus (HCV) infection is an immune complex-mediated type of systemic vasculitis, preferentially involving the small vessels (34). *MX1* may be associated with interferon signaling and have an essential role in the cellular antiviral response that is involved in systemic vasculitis, which requires further investigation.

Furthermore, the present study demonstrated that the predicted TFs, *STAT1*, *MAFB*, and *YWHAZ*, which interacted with each other, regulated further DEGs as target genes in the regulation network, indicating the importance of them. The protein encoded by *STAT1* is a member of the STAT protein family, which are phosphorylated by receptor-associated kinases, and subsequently act as transcriptional activators. Previous studies have demonstrated that STAT acts as a signal transducer and transcriptional activator, and mediates cellular responses to interferons, other cytokines and growth factors (35-37). Lin *et al* (38) demonstrated that HCV suppressed interferon signaling by degrading *STAT1*. Chan *et al* (39) reported that type I interferons, when used to treat resistant Churg-Strauss syndrome, a type of systemic vasculitis, led to complete remission in 25% of a small case series. In a previous study, it was observed that *MAFB* suppressed acute inflammatory responses in lipopolysaccharide-stimulated lung injury (40). Additionally, it was demonstrated that *MAFB* modulated the efficiency of interferon production. On the other hand, *YWHAZ* belongs to the 14-3-3 family of proteins, which mediate signal transduction and gene regulation events through binding to phosphoserine-containing proteins (41). Nishimura *et al* (41) demonstrated that overexpression of *YWHAZ* may inhibit cell apoptosis in breast cancer cell lines. In addition, the activation of *STAT1* was demonstrated to induce apoptosis (42). Furthermore, Jamin *et al* (43) revealed that apoptosis of endothelial cells was induced by the binding of anti-endothelial cell antibodies to heat shock protein family D (Hsp60) member 1 in vasculitis-associated systemic autoimmune diseases. We hypothesized that *STAT1* may be involved in interferon signaling transduction via interaction.
with MAFB, and STAT1 may participate in cell apoptosis through interaction with YWHAZ in systemic vasculitis.

In conclusion, the critical genes involved in systemic vasculitis have been investigated based on the microarray data used in this study. FOS may function in TLR signaling that is involved in the inflammatory response and UBB may function in the progression of systemic vasculitis via participation in the Notch signaling pathway. In addition, MXI may be associated with interferon signaling and have an essential role in the cellular antiviral response in systemic vasculitis. Furthermore, STAT1 may be involved in interferon signaling transduction via interaction with MAFB, and STAT1 may participate in cell apoptosis through interaction with YWHAZ in systemic vasculitis. Further experiments and investigation with larger sample sizes are required to verify these results. Insights into promising gene targets should lead to novel and effective strategies, and improved targeted therapy for systemic vasculitis.

Acknowledgements

This study was supported by the Shanghai Science and Technology Commission fund (grant no. 15495810202), Medical Engineering Cross Research fund (Shanghai Jiaotong University; grant no. YG2013MS08) and Medical Engineering Cross Research fund (Shanghai Jiaotong University; grant no. YG2014QN09).

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