RNA-seq analysis of molecular heterogeneity peripheral blood mononuclear cells in systemic lupus erythematosus

Ke Xue[1,2], Ya-Jie Zheng[3], Rui-Xing Yu[1], Xue Shen[3], Yong Cui[1,2]

[1]Department of Dermatology, China-Japan Friendship Hospital, Beijing 100029, China;
[2]Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China;
[3]Institute of Dermatology and Department of Dermatology, the First Affiliated Hospital, Anhui Medical University, Anhui, Hefei 230022, China.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a broad spectrum of clinical manifestations. Although great progress has been made in understanding the contribution of genetic and environmental factors in SLE pathogenesis, as there are over 20,000 published studies evaluating related cellular dysfunction,[1] the exact mechanisms that underline this process are still unknown. Over the past 10 years, novel insights have mainly originated from genome-wide association (GWAS) and gene expression studies.

It is known that SLE is characterized by chronic inflammation, and that nearly all lupus patients exhibit increased levels of anti-nuclear antibodies in peripheral blood.[2] In addition, type I alteration of the interferon (IFN) system, the presence of auto-reactive T- and B-cells and inefficient clearance of apoptotic cell debris by monocytes/macrophages, are also hallmarks of SLE.[3] Apoptotic debris are believed to cause much of the type I IFNs signature and type I IFNs themselves can drive additional apoptosis. Nevertheless, much remains unknown about the pathogenesis of SLE, particularly at the level of nucleic acid accumulation and dysregulation of gene expression. Dysregulated gene expression, with the accumulation of aberrant transcripts, could theoretically contribute to apoptosis or increased type I IFNs expression and has been shown to mimic lupus.[4] However, all of these data were generated through microarray analysis of whole blood or peripheral blood mononuclear cells (PBMCs) from different SLE patients, resulting in an unclear global view of the dysregulated gene signature.

Recently, gene expression profiles from specific cell subsets have been shown to be better determinants of immune status than bulk PBMC profiles due to the diversity of leukocyte responses.[5] In this study, we explored the transcriptional signature of PBMCs from clinically and pathologically well-characterized patients with mild to severe SLE and establish its correlation with disease progression. A global landscape of dysregulated gene signatures was firstly obtained using high-throughput RNA sequencing, followed by pathway analysis and subsequent immune profiling to show differential responses across different cell types. These findings could provide insights into the heterogeneity of the disease, and identify biomarkers as well as pathways driving disease pathogenesis.

A total of nine SLE patients and eleven normal healthy volunteers (NHVs) were recruited from the outpatient department at Department of Dermatology, China-Japan Friendship Hospital from 2016 to February 2018. Informed written consent was obtained from all participants enrolled in this study and this study was performed in accordance with the 1964 *Declaration of Helsinki* and its later amendments. We divided the patients into two groups based on their SLE Disease Activity Index (SLEDAI) score. Patients in group 1 had mild disease, including patients with a SLEDAI score ≤ 9; group 2 was composed of patients with severe disease activity, or SLEDAI > 9. All patient information is listed in Table 1. Briefly, 5 mL of peripheral blood was collected in sterilized, heparin-coated tubes. PBMCs were isolated from whole blood in cell preparation tubes (Becton–Dickinson) and were stored at −80°C until use. All SLE patients did not take any medication before the PBMC sample was taken.

Total RNA was extracted from PBMCs, purified T cells or monocytes using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Samples were stored at −80°C until further use. RNAs extracted from

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Correspondence to: Dr. Yong Cui, Department of Dermatology, China-Japan Friendship Hospital, Beijing 100029, China
E-Mail: wuhucuiyong@vip.163.com

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Table 1: Characteristics of the patients with systemic lupus erythematosus and normal healthy volunteers.

Characteristics	NHV ($n=11$)	SLE ($n=9$)	Mild ($n=4$)	Severe ($n=5$)
Sex, male/female	0/11	0/9	0/4	0/5
Age (years), mean ± SD	32.0 ± 4.7	32.8 ± 10.2	37.7 ± 8.2	29.8 ± 10.2
SLEDAI score*, mean ± SD	NA	9.8 ± 4.9	5.0 ± 0.8	12.8 ± 3.9

Disease Activity Index score (score of 5–9 were considered mild; scores of ≥10 were considered severe). *Disease activity was classified as mild and severe according to the systemic lupus erythematosus. NA: Not available; NHV: Normal healthy volunteers.

NHV and SLE PBMCs were used for commercial RNA-seq analysis (Beijing Genomics Institute, BGI, Beijing, China).

RNA-seq was performed on extracted RNA from both SLE patients ($n=9$) and NHV ($n=11$). In total, 20 peripheral blood samples were obtained from nine SLE patients, four mild and five severe, and 11 from NHV. Globin RNA was removed from blood samples using Globin–Zero Gold rRNA removal kit. Then, extracted PBMC RNA was purified using RNeasy MinElute Cleanup and PAXgene Blood miRNA Kits per the manufacturer's instructions. The integrity of extracted RNA was assessed using RNA 6000 Nano Kit on Agilent 2100 Bioanalyzer and RNA sequencing was performed using Illumina HiSeq 2000 (BGI platform).

All statistical analyses were performed in GraphPad Prism software (GraphPad Software, San Diego, CA, version 6.0). Unpaired *t* test with or without Welch correction was used to compare different groups and Spearman rank correlation was used to calculate the correlation between two groups. Statistical significance was set at a $P<0.05$.

SLE patients experience episodes of progression, remission and relapse. In an attempt to identify the genes associated with disease severity index, a gene profiling approach was employed. We collected RNA from SLE patients ($n=9$), including four mild and five severe SLE patients, as well as NHV ($n=11$). RNA-seq assay was used to screen the whole transcriptional profile. Principal component analysis (PCA) revealed that the differences in the expression profiles of NHV and SLE are distinct while variation of mild and severe samples are relatively small [Figure 1A]. Such observations were further confirmed via creation of a heatmap [Figure 1B]. Notably, the correlation heatmap also suggested two SLE samples (sle1 and sle9) had different correlations compared with other samples. Taken together, SLE patients PBMC showed different gene expression profiles in contrast with NHV. To further elucidate which genes were affected during SLE disease development and progression, genes specifically up- and down-regulated were analyzed. Differential expression analysis demonstrated that 2146 genes were differentially expressed between disease and NHV groups, among which 1040 genes had higher expression in SLE patients and 1106 genes had higher expression levels in healthy individuals [Figure 2A]. To identify the gene expression pattern related to the disease severity, we compared transcriptomes from mild and severe groups against the normal group. In mild *vs*. normal, 1662 DEGs were found, with 739 up-regulated and 923 down-regulated; whereas severe *vs*. normal identified 2350 DEGs, 1137 up-regulated, 1213 down-regulated. However, only 50 DEGs were obtained after a comparison of severe *vs*. mild (33 up-regulated, 17 down-regulated) with no significance as the samples were scattered. Therefore, significant differential expression was observed between SLE and NHV, but not for the severe and mild disease comparison. Further, our DEG heatmap had a similar pattern as single gene analysis [Figure 2B], in which mild and severe SLE patient gene expression pattern could not be distinguished with each

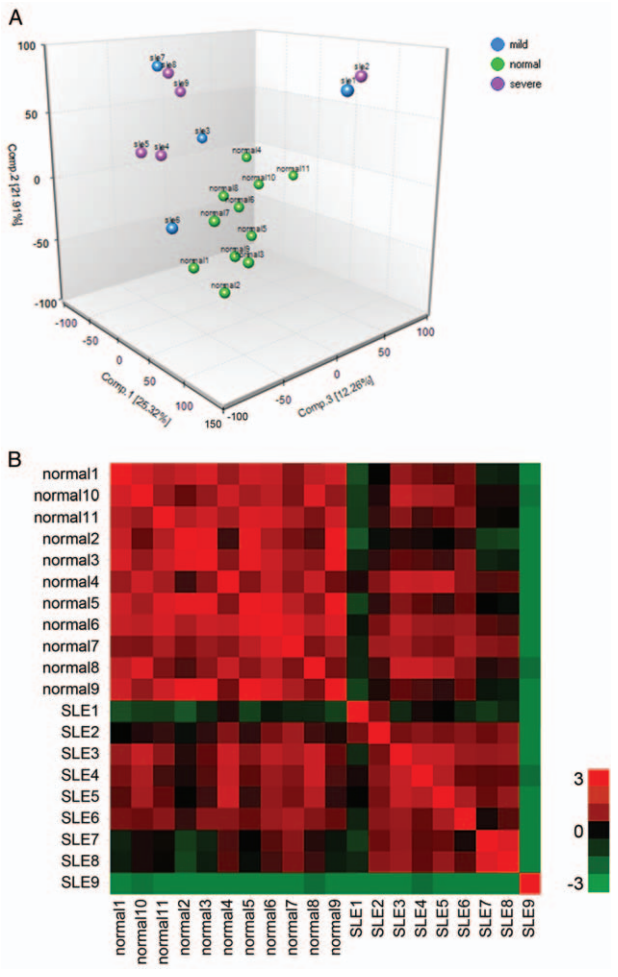

Figure 1: PCA analysis and correlation analysis for RNA from normal donor and SLE patients. (A) PCA analysis showed that normal samples are different from disease samples, however, mild and sever samples are hard to distinguish. (B) The correlation heatmap showed that all sample except sle1 and sle9 showed good correlations compared with other samples. Red indicates genes expressed at higher levels relative to the control mean, and green represents genes expressed at lower levels than control mean. The correlation heatmap showed that sle1 and sle9 showed different correlations compared with other samples. PCA: Principal component analysis; SLE: Systemic lupus erythematosus.

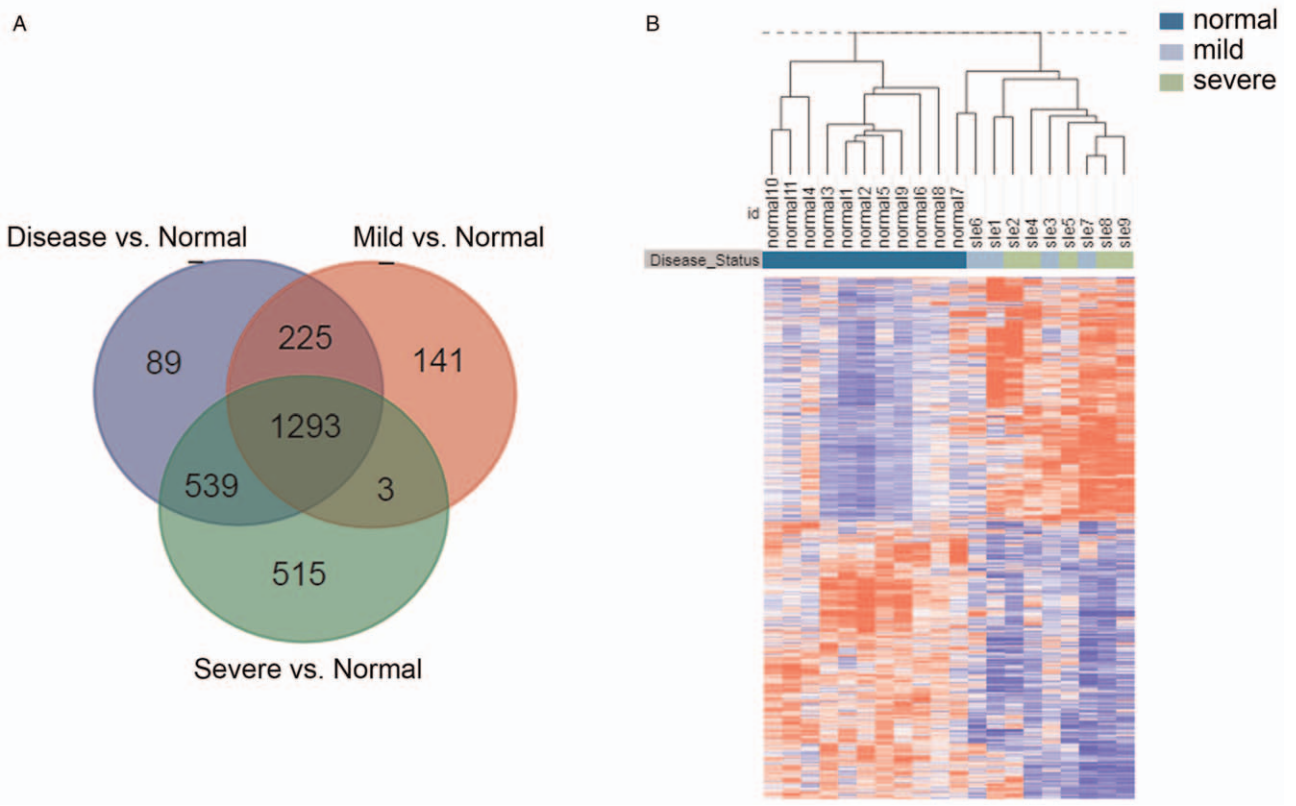

Figure 2: Different expressed gene (DEG) profile investigation using RNA-seq. (A) Significant genes were selected with cutoff, $P < 0.05$, Fold change > 2.0. Disease *vs.* Normal comparison, 2146 DEGs, 1040 up-regulated, 1106 down-regulated; Mild *vs.* Normal: 1662 DEGs, 739 up-regulated, 923 down-regulated; Severe *vs.* Normal: 2350 DEGs, 1137 up-regulated, 1213 down-regulated; Severe *vs.* Mild: 50DEGs, 33 up-regulated, 17 down-regulated. (B) heatmap analysis for significant changed genes. Normal, mild, and severe were labeled with different color.

other. Interestingly, a majority of the top differentially expressed genes overlapped between mild *vs.* normal and severe *vs.* normal comparisons. These findings indicate the gene expression pattern in mild and severe SLE is similar.

Gene expression studies of PBMCs from patients with SLE have determined the presence of a type I IFNs signature and increased expression of inflammatory cytokine genes.[6] However, detailed analysis was not performed in these reports. In our study, we analyzed PBMC gene expression patterns comparing SLE patients and normal healthy controls. These data were comparable to the findings of other studies, which indicated major changes, including the type I IFNs pathway, are a product of the immune response. The transcriptional profile of immune cells can help us understand the heterogeneity of disease, and it has been reported in the literature that dendritic cells are altered in autoimmune patients and are associated with disease activity.[7] Interestingly, we found comparable changes in the number of up-regulated genes and down-regulated genes, for example, comparing disease *vs.* normal (NHV), 1040 up-regulated and 1106 down-regulated [Figure 2A]. Additionally, the same pattern was found in heatmap analysis for differentially expressed genes [Figure 2B]. Using gene enrichment analysis, we observed the up-regulated and down-regulated gene sets affected different pathways, such as the immune response and cell cycle. This indicates differentially expressed gene sets affect different functional pathway in the peripheral blood of SLE patients.

Besides of gene expression pattern change analysis, SLE disease scoring was also considered as a potential factor regulating gene expression in PBMCs among patients. According to this hypothesis, we compared significantly different expressed genes in severe *vs.* normal and mild *vs.* normal sample groups. results showed that there was no significant difference within each comparison. Additionally, few genes were found when mild *vs.* severe samples were compared, which had only 50 DEGs with 33 up-regulated and 17 down-regulated.

In support of this finding, PCA and heatmap results showed a similar pattern, in which the mild and severe group could not be distinguished from each other. After carefully checked our scoring for SLE patients, we reasoned the grouping of severe and mild SLE patient may not reflect the significant change in gene expression. These data suggest that SLE disease grouping could not change gene expression pattern in PBMCs of SLE patients. The expression of many genes in lupus patients with different disease activity levels is indistinguishable. According to our analysis results, 50 differential genes can be screened out. The RNA-seq data analysis enriched the differential genes and found that a large number of differential genes were mainly enriched in the IFN signaling pathway. In addition, some differential genes

can also involve in JAK/ STAT, MAPK-related pathways, and more than 50 articles have been searched for these pathways.[8]

In this study, we identified aberrant expression profiles of PBMCs from SLE patients. This study expanded our understanding of the genetic molecular mechanisms in SLE and may contribute to the discovery of potential biomarker for the assessment and diagnosis of SLE patients.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

None.

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