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Original Paper

A Time-Series Analysis of Severe Burned Injury of Skin Gene Expression Profiles

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Key Words
Geo • Burned • Soft clustering • DAVID • R

Abstract
Background/Aims: Major burn injury is one of the main severe forms of wound which lead to a mass of clinical debilitation, this study was to identify core biomarkers for the recovery of severe burned injury. ***Methods:*** Gene expression profiles (GSE19743) from the Gene Expression Omnibus (GEO) was downloaded, followed by background correction, normalization of raw microarray dataset and identification of differential expression genes (DEGs) . Soft clustering of DEGs was used for the screening of gene clusters that with sustained increasing (SIG) and decreasing expression (SDG) profiles along with the recovery process of burned injury. The significantly enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of SIGs and SDGs were obtained through the Database for Annotation, Visualization, and Integrated Discovery (DAVID), based on which the miRNA-gene regulation network for SIGs and SDGs were constructed from the miRWalk database. ***Results:*** Ten clusters were obtained through soft clustering. The SIGs and SDGs were found to be closely associated with the biological processes of immune system. The miRNA-gene regulation network analysis suggested different roles between SIGs and SDGs in the recovery of severe burned injury. Furthermore, a bunch of important biomarkers were identified, which would be helpful in the treatment of burned patients. ***Conclusion*:** Our current findings suggest an interesting molecular link between transcriptional regulation potentially involved in immunosuppressive state after major burn injury, which warrants further exploration for their utilization in the treatment of major burn injury.

H.-T. Xu and J.-C. Guo contributed equally to this work.

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Introduction

Major burn injury is one of the main severe forms of wound which lead to a mass of clinical debilitation coupled with economic influence, influencing up to millions of injuries and deaths per year in the world. In addition to the devastation of the skin barriers protection by major burn injury, one of the most complications involving the clinical course of recovery is generalized immunocompromise that further caused recurrent inflammation and prolonged healing. Twenty years ago, Wolfe et al. noticed that immunosuppressive serum and an impaired lymphocyte response are associated with anergy in burn patients and confirm that the development of anergy is an index of poor prognosis [1]. Then, several attempts have been made to clarify the relationship of immunosuppressive and burn injury. Barrow and his colleague found that burn injury induced a significant increase in serum TGF-beta, which may contribute to post-burn immunosuppression with an increased susceptibility to sepsis [2]. Another study have demonstrated that the productive capacity of macrophages for inflammatory mediators, including nitric oxide, prostaglandins, *TNF-α* and *IL-6*, is profoundly increased post-burn, thereby implicating macrophages in the development of the post-burn immunosuppression [3]. What's more, production of interleukin-10 has also been shown to be increased by cells of the immune system in human and mouse after burn injury [4]. All of these studies indicated the importance to explore the mechanisms of immunosuppressive and burn injury. Despite recognized roles for immunosuppressive in regulating healing and infections of burn injury, our mechanistic understanding of immunocompromise and its systemic influence, and our capacity to manipulate this response are still limited.

In this study, gene expression profiles (GSE19743) were downloaded from the Gene Expression Omnibus (GEO). They collected leukocytes from blood samples of severe burned patients and healthy controls at different times after severe burn injury. Total cellular RNA was isolated from the leukocyte pellets and gene expression was measured using Affymetrix U133 Plus 2.0 arrays. Background correction, normalization of raw microarray dataset and identification of differential expression genes (DEGs) were conducted via R. Soft clustering of DEGs was used for the screening of gene clusters that with sustained increasing (SIG) and decreasing expression (SDG) profiles with the recovery of burned injury. What's more, SIGs and SDGs were uploaded to David online database to identify significantly enriched GO terms and KEGG pathways. Then we focus on genes associated with transcription control. In conclusion, our finding has explained the central importance of understanding immunosuppressive state after major burn injury and provide potential therapeutic targets for further studies.

Materials and Methods

Gene Expression Datasets

We obtained the gene expression profiles from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) in NCBI with the accession number of GSE19743[5]. A total of 177 blood samples of 57 severe burned patients with two biological replicates and 63 healthy controls with one replicate were contained in the dataset. In this study, we divided the samples into four groups, i.e. healthy controls, patients of 0~100 hours, 100~400 hours and longer than 400 hours after severe burn injury, and they were referred as 'H', 'E', 'M' and 'L' respectively hereafter.

Microarray Analysis

All of the analysis processes of the gene expression microarray were conducted in R. Briefly, raw microarray datasets in CEL were imported into R and a Robust Multi-Array (RMA) method was adopted for the background correction and normalization through *affy* package [6]. Then, we identified the differential expression genes (DEGs) in the 114 burned patients compared with the 63 healthy controls via *limma* package [7] with the criteria of fold change > 2 and FDR adjusted *P-Value* < 0.05.

Soft Clustering of DEGs

We considered genes with sustained increasing or decreasing expression profiles with the recovery of injury as those important ones. So one of the core processes is to identify those genes. In this study, we conducted soft clustering through *Mfuzz* package [8], which could allocate a gene into multi clusters according to its clustering coefficient (CC). Here, we screened out the gene clusters with sustained increasing and decreasing profiles from H group to L group, i.e. H – E – M – L through the soft clustering of DEGs.

Functional Enrichment Analysis

To explore the enriched functions of genes in sustained increasing (SIG) and decreasing (SDG) clusters, functional enrichment analysis was conducted in the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/)[9]. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with *P-Value* < 0.05were obtained.

MiRNA-Gene Regulation Network

MicroRNA (miRNA) is a small non-coding RNA molecule containing about 22 nucleotides. Through regulating the expression of its target genes, it could affect the progression of many types of diseases, such as cancer. While very few studies have focused on the influences of miRNA in the recovery of burned injury. In this study, we screened out miRNAs that might regulate SIGs and SDGs through miRWalk database [10]. Only the miRNA-gene pairs exist in all of the TargetScan, miRanda, RNA22, PITA and miRWalk databases were considered reliable. Besides, the miRNA-gene regulation network was visualized through Cytoscape [11].

Results

Differential Expression Analysis

The normalized expression values were used for the identification of DEGs. With the criteria of fold change > 2 and FDR adjusted *P-Value* < 0.05, a total of 1215 DEGs were identified in the 114 burned samples compared with the 63 healthy controls.

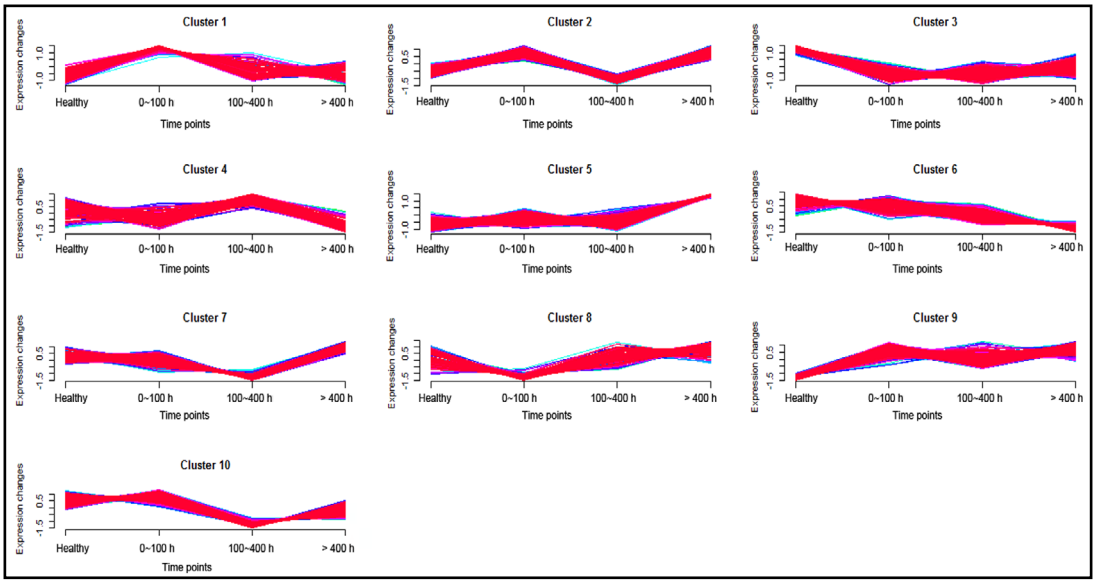

Fig. 1. The 10 clusters obtained through soft clustering.

SIGs and SDGs

Soft clustering of the 1215 DEGs resulted in 10 clusters (Fig. 1), and among which, most of the genes in cluster 6 were found to be with sustained decreasing expression profiles from H to L and the genes in cluster 8 with sustained increasing expression profiles. Thus, genes in cluster 6 and cluster 8 with CC > 0.5 were considered as SDGs and SIGs respectively. The top 10 SDGs and SIGs with the highest score in cluster 6 and cluster 8 were shown in Table 1.

For other clusters, for example cluster 1 and cluster 2, whose expression profiles were firstly up-regulated followed by down-regulated. Meanwhile, some clusters have inverse expression regulation trend, for example cluster 3 and cluster 4, in which the gene expression values were firstly down-regulated followed by up-regulated.

Table 1. Top 10 genes with the highest scores in cluster 6 and cluster 8

Cluster 6	Score	Cluster8	Score
LMO7DN	1.000	DPEP2	0.999
CISD3	1.000	NOG	0.999
ADAM28	0.999	CBFA2T3	0.999
ABCB1	0.999	ZNF76	0.999
TRPC1	0.999	TRPM2	0.995
MRPS25	0.999	ADGRE3	0.995
QDPR	0.999	ALKBH2	0.993
MS4A1	0.997	ANKRD23	0.992
CARNS1	0.994	WDR60	0.992
E2F2	0.993	B3GAT1	0.990

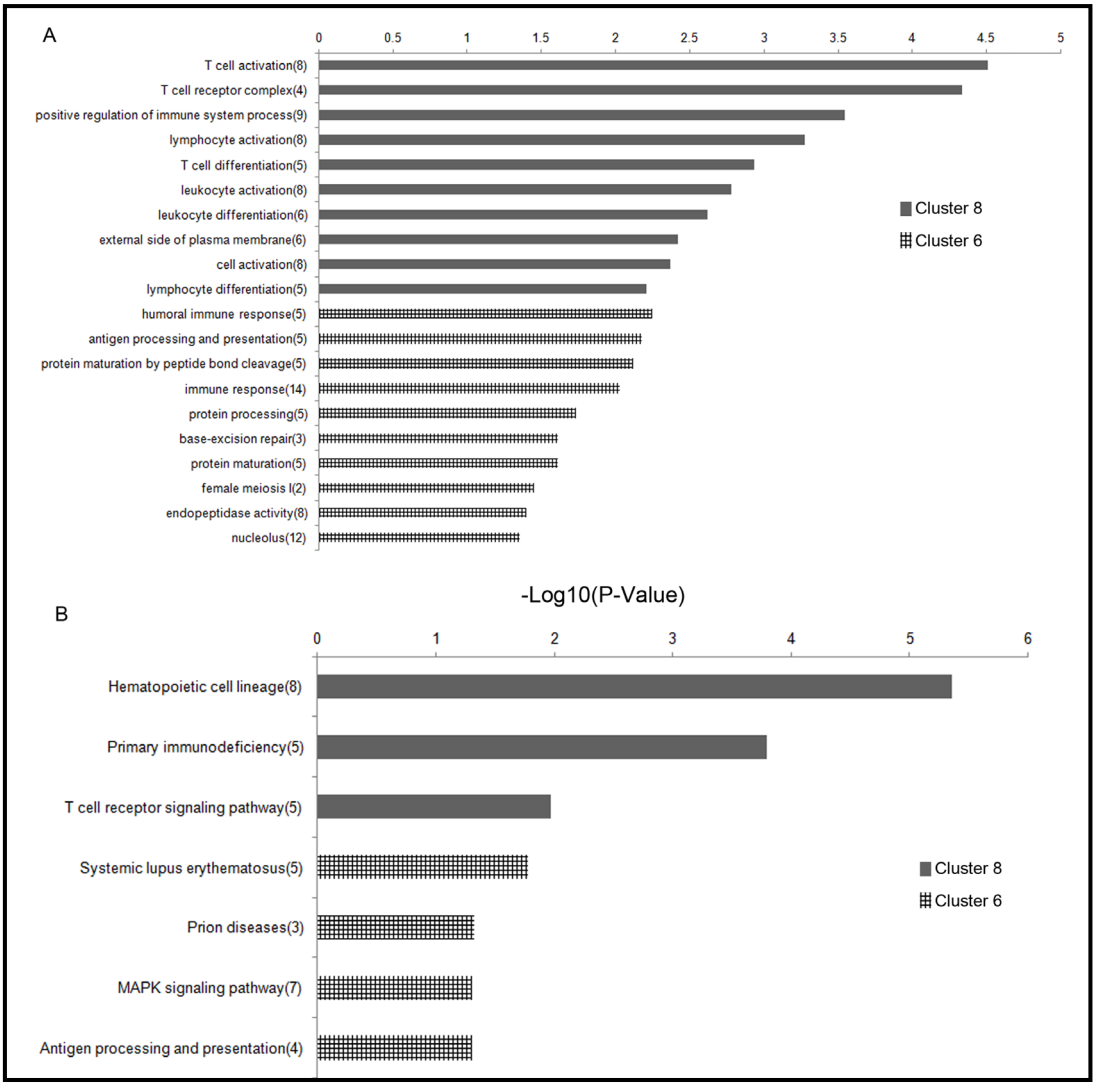

Fig. 2. The top 10 most significant GO terms (A) and all of the significantly enriched KEGG pathways (B) for SIGs and SDGs.

Enriched Functions

A total of 89 and 76 enriched GO terms were obtained for SDGs and SIGs respectively with the criteria of *P-Value* < 0.05. Most of those terms were found to be associated with immune response, such as T cell activation, T cell complex receptor, humoral immune response, antigen processing and presentation. Besides, there were four and three KEGG pathways were significantly enriched in SIGs and SDGs respectively, which also mainly involved in the biological processes of immune system. Fig. 2A and 2B illustrated the top 10 significantly enriched GO terms and all of the KEGG pathways for SIGs and SDGs.

MiRNA-Gene Regulation Network

The miRNA-gene regulation networks for SDGs and SIGs were shown in Supplemental Fig. S1 and Supplemental Fig. S2 respectively (For all supplemental material see www.karger.com/10.1159/000493451/). The distribution of degree (number of nodes directly link with one node) of the two miRNA-gene regulation network were shown in Fig. 3A and B and compared through wilcoxon test. As a result, degrees in the miRNA-gene regulation network of SIGs were significantly higher than that of SIGs' (Fig. 3C, *P-Value* = 0.0485). Besides, Table 2 showed the top 10 miRNAs with highest degree in the two networks.

Table 2. The top 10 miRNAs with the largest number of targets in the miRNA-Gene network of cluster 6 and cluster 8

Cluster 6	Degree	Cluster8	Degree
miR-325-3p	17	miR-23-3p	12
miR-29	16	miR-181-5p	11
miR-29-3p	16	miR-506	10
miR-29bc	16	miR-340-5p	10
miR-124	15	miR-325-3p	10
miR-29bc-3p	14	miR-27-3p	10
miR-27	13	miR-25	10
miR-27-3p	13	miR-23	10
miR-302	13	miR-204	10
miR-23-3p	12	miR-181	10

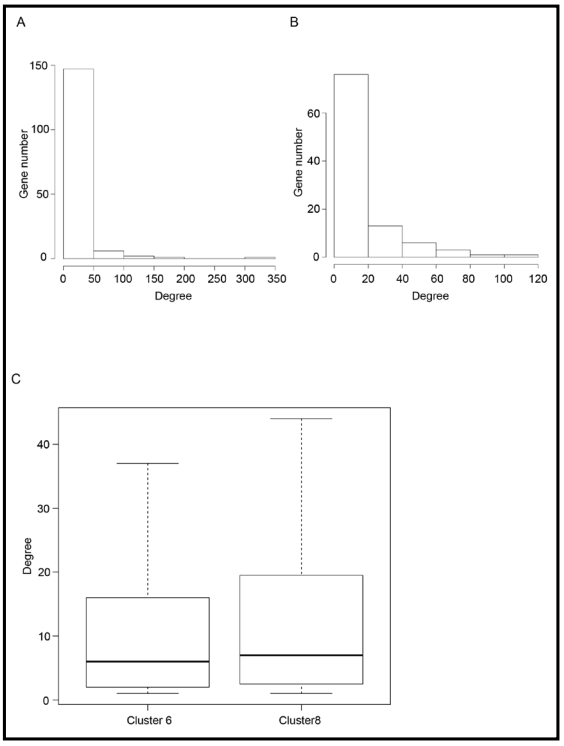

Fig. 3. The degree distribution of miRNA-gene network of SIGs (A) and SDGs (B) and the comparison of those two lists of degrees (C).

Discussion

In the past two decades, a number of researchers have reported that major burn injury causes an immunosuppressive state that predisposes victims to subsequent sepsis and multiple organ failure. A considerable amount of literature has been published on immunosuppressive mechanisms, including dominance of inhibitory over activating receptors, expansion of suppressive cell types, as well as pro-inflammatory and anti-inflammatory cytokines injury. However, far too little attention has been paid to transcriptional regulation of immunosuppressive state in major burn injury study. The fungal metabolites cyclosporin A (CsA) and tacrolimus (FK506),

which inhibit the calcium-dependent serine/threonine phosphatase calcineurin and its substrate, the transcription factor *NFAT*, are among the most potent immunosuppressive drugs available today. In addition of *NFAT*, a number of transcription factors have reported to control immunosuppression. For example, transcription factors *Stat3* and *Gfi-1* could control Th17 cell immunosuppressive state by induced the expression of ectonucleotidases. What's more, *TGF-β* induced *Foxp3* gene expression in TCR-challenged $CD4^+CD25^-$ naive T cells, which mediated their transition toward a regulatory T cell phenotype with potent immunosuppressive potential. All of these researches implied that transcription factor may be potent drug target for immunosuppressive therapy.

To understand the cell and molecule mechanism response to burn injury, leukocytes from blood samples of severe burned patients and healthy controls were collected at different times after severe burn injury. After background correction, normalization of raw microarray dataset and identification of differential expression genes (DEGs), a total of 1215 DEGs were identified. Soft clustering of DEGs was used for the screening of gene clusters that with SIG and SDG profiles with the recovery of burned injury. The significantly enriched GO terms and KEGG pathways of SIGs and SDGs were obtained through DAVID online database. In accordance with other research, significantly enriched ontology categories were found to be closely associated with immune activation, including immune response, T cell differentiation, T cell costimulation, adaptive immune response and positive regulation of calcium-mediated signaling and etc. Interestingly, there is another GO term associated with transcriptional control drawn our attention. We noticed that the expression level of some transcription factors, including *ELP2*, *FOXO6*, *ZNF18*, *ZNF76*, *POU3F4*, *TEAD3* and *SUPT3H* were sustained increased post burn injury. It is reasonable to believe that the up-regulation of these transcription factors maybe drivers or at least played a critical for inducing the immunosuppressive state. *ELP2* is a core subunit of the elongator complex effected transcriptional elongation and may help remodel chromatin. Mou and his colleagues showed that Elongator subunit 2 (*ELP2*) regulated the kinetics of defense gene induction and accelerated immune responses [12]. *FOXO6* is an important paralog of *FOXO1*. Recent studies have highlighted a fundamental role for Forkhead box O (FOXO) transcription factors in immune system homeostasis [13]. We believe that these genes may be critical mediators in pathogenesis of immunocompromise which finally manipulate poor prognosis.

In this study, we also conducted miRNA-gene regulation analysis for SIGs and SDGs. MiR-23-3p has the most SDGs targets than other miRNAs in miRNA-gene regulation network of SDGs. In Pedrera's study [14], miR-23-3p was found to be significantly up-regulated by anti-TNFα in rheumatoid arthritis patients, which indicated its role in immune response. Here, we demonstrated that post-burn immunosuppression was proved to contribute burn recovery, so miR-23-3p might also involve in the biological processes post-burn. MiR-29 is the second miRNA with the most gene targets in miRNA-gene regulation network of SIGs. There are several studies indicated the important role of miR-29 in immune response [15-17], so miR-29 should also be an important biomarker for burn recovery. It is important to take miRNA into account for the discovery of novel drugs for severe burn treatment.

Conclusion

All in all, by the identification of DEGs, clustering, GO enrichment, KEGG pathway analysis, some key genes that might play important roles in immunocompromise were screened out. And our current findings suggest an interesting molecular link between transcriptional regulation potentially involved in immunosuppressive state after major burn injury. However, the results of our study remains to be further explored for their utilization in the treatment of major burn injury.

Disclosure Statement

The authors declare to have no competing interests.

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