Differential gene expression analysis reveals novel genes and pathways in pediatric septic shock patients

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Septic shock is a devastating health condition caused by uncontrolled sepsis. Advancements in high-throughput sequencing techniques have increased the number of potential genetic biomarkers under review. Multiple genetic markers and functional pathways play a part in development and progression of pediatric septic shock. We identified 53 differentially expressed pediatric septic shock biomarkers using gene expression data sampled from 181 patients admitted to the pediatric intensive care unit within the first 24 hours of their admission. The gene expression signatures showed discriminatory power between pediatric septic shock survivors and nonsurvivor types. Using functional enrichment analysis of differentially expressed genes, we validated the known genes and pathways in septic shock and identified the unexplored septic shock-related genes and functional groups. Differential gene expression analysis revealed the genes involved in the immune response, chemokine-mediated signaling, neutrophil chemotaxis, and chemokine activity and distinguished the septic shock survivor from non-survivor. The identification of the septic shock gene biomarkers may facilitate in septic shock diagnosis, treatment, and prognosis.

The pediatric septic shock dataset contains gene expression profiles of peripheral blood samples collected from 181 patients including 154 survivors and 27 non-survivors within the first 24 hours of admission. Overall mean age of the patient cohort was 3.69 years with a standard deviation of 3.28. Mean age of the survivors was 3.78 years (standard deviation of 3.15), compared to 3.19 (standard deviation of 3.93) for the non-survivors. Males (109;
60.2%) had higher representation than females (72; 39.8%). Of the 27 non-survivors, 19 were male and only 8 were female; whereas, in the non-survivor group the male and females were 90 and 64, respectively. Clinical characteristics (such as mortality, organism, source of infection, malignancies, immunosuppression, gram-positive bacteria, and acute kidney injury) of the 181 patients are included in Supplementary Dataset 1.

Etiologic diagnosis was identified for 107 (59.12%) patients. As shown in Table 1, the most frequently identified pathogens were Streptococcus pneumoniae (in 17 cases), Staphylococcus aureus (in 17 cases), and Streptococcus pyogenes (in 12 cases). Detailed clinical characteristics including source of infection and organism are given in Supplementary Dataset 1.

Identification of differentially expressed upregulated and downregulated genes. Based on preset criteria of an adjusted p-value < 0.05, a total of 53 genes from 21,731 were shown to be differentially expressed genes (DEG) between Survivor and Non-survivor types, including 47 genes that were upregulated and 7 genes that were downregulated. Those DEGs with a fold change of at least 1.5 (n = 16) is shown in Table 2 (For complete list, refer to Supplementary Dataset 1).

Functional enrichment analysis of differentially expressed genes. 53 DEGs were analyzed by KEGG pathway and Gene Ontology (GO) term enrichment. A total of 52 genes were recognized in the DAVID database. KEGG pathway analysis revealed rheumatoid arthritis (RA) (hsa: 05323), and cell cycle (hsa: 04110) pathways as the most significant pathways (Table 3). GO analyses of the DEGs demonstrated that mitotic sister chromatid segregation (GO: 0000070), immune response (GO: 0006955), cell division (GO: 0051301), and chemokine-mediated signaling pathway (GO: 0070098) were the most enriched biological process (BP) terms (Table 3). Chemokine activity (GO: 0008009) was the most enriched term under molecular function (MF) (Table 3). Chemokine interleukin-8-like domain (IPR001811), CC chemokine, conserved site (IPR000827) InterPro protein functional groups were among the significantly enriched functional classes associated with septic shock development (Table 3).

Figure 1 illustrates a heatmap of 53 selected DEGs in survivor and non-survivor groups. The vertical axis represents patient samples (see Supplementary Dataset 1 for more details), and the horizontal axis represents DEGs with the log2 gene expression intensity values. Red and green represent the upregulated, and downregulated genes, respectively. The heatmap is clustered using both samples and genes categories. Right vertical bars are used for annotation of mortality, pathogen, gram-positive, immunosuppression, gender status, and infection source.

| Etiology                          | Number of patients | Total (%) |
|-----------------------------------|--------------------|-----------|
| Streptococcus pneumoniae          | 17                 | 9.39      |
| Staphylococcus aureus             | 17                 | 9.39      |
| Streptococcus pyogenes            | 12                 | 6.63      |
| Klebsiella                        | 9                  | 4.97      |
| Streptococcus agalactiae          | 7                  | 3.87      |
| Neisseria meningitidis            | 7                  | 3.87      |
| Enterococcus                      | 5                  | 2.76      |
| Gram-negative rods                | 4                  | 2.21      |
| Simplexviruses                    | 3                  | 1.66      |
| Influenza A virus                 | 3                  | 1.66      |
| Mixed                             | 3                  | 1.66      |
| Enterobacter                      | 2                  | 1.11      |
| Hemophilus                        | 2                  | 1.11      |
| Pseudomonas                       | 2                  | 1.11      |
| Serratia                          | 2                  | 1.11      |
| Acinetobacter                     | 1                  | 0.55      |
| Adenovirus                        | 1                  | 0.55      |
| BK virus                          | 1                  | 0.55      |
| Candida                           | 1                  | 0.55      |
| Clostridium                       | 1                  | 0.55      |
| Cytomegalovirus                   | 1                  | 0.55      |
| Escherichia coli                  | 1                  | 0.55      |
| Group F Streptococcus             | 1                  | 0.55      |
| Human metapneumovirus             | 1                  | 0.55      |
| Moraxella                         | 1                  | 0.55      |
| Parainfluenza                     | 1                  | 0.55      |
| Rickettsia                        | 1                  | 0.55      |
| Unknown                           | 74                 | 40.88     |

Table 1. Etiology of septic shock in 181 patients.
There are two major sample clusters at the top level of the dendrogram. Only 4 (15%) out of 27 non-survivors were in the first cluster, while the remaining 85% formed the second cluster. Similarly, for the gram-positive category, samples formed two clusters. We observed no clear trends or cluster formation for the other annotation groups. Genes were clustered into two major groups of downregulated genes (MT1M to CFH except for DDIT4 and RGL1), while the remaining genes clustered in the upregulated category. Overall, DDIT4, NDUFV2, and TNFRFS10C were the most highly expressed genes, whereas AREG, CCL20, and CFH were the least expressed genes.

**Discussion**

This study of peripheral blood mRNA sequences revealed key genes and functional characterization associated with septic shock survival and non-survival. From the differential gene expression analysis, we identified potential septic shock biomarkers that may help in an unbiased septic shock diagnosis and effective treatment, and ultimately improving prognoses.

Differentially expressed gene analysis using septic shock samples provides insights into the functional characterization of genes between groups of septic shock survivor and non-survivor samples. However, like any other, microarray data analysis is incomplete without performing adjustment for multiple testing. Due to approximately 20,000 (approximate number of genes on a standard microarray chip) independent tests, it is expected to get at least 20 test scores by random chance when we allow a stricter p-value threshold of, for example 0.001. To avoid this situation, adjustment for multiple testing was utilized, and we used the Benjamini Hochberg method. For a false-discovery rate (FDR) controlling procedure, adjusted p-value of an individual hypothesis is the minimum value of FDR for which the hypothesis is first included in the set of rejected hypotheses, and we used an adjusted p-value cut-off of 0.05.

**Table 2.** List of most significant upregulated and downregulated genes in septic shock.

| Gene   | Fold Change | Average Expression | t-statistics | p-value       | adj. p-value | Reference |
|--------|-------------|--------------------|--------------|---------------|--------------|-----------|
| DDIT4  | 2.12        | 8.441545           | 5.490723     | 1.33E-07      | 0.080592     | 24        |
| CCL3   | 2.12        | 6.208106           | 4.627029     | 7.02E-06      | 0.012717     | 24        |
| PRG2   | 2.11        | 5.48492            | 5.611777     | 7.35E-08      | 0.000533     | —         |
| MT1M   | 1.78        | 3.72997            | 5.641576     | 6.35E-08      | 0.000533     | —         |
| CDC20  | 1.68        | 6.18643            | 4.059008     | 7.31E-05      | 0.040893     | 24        |
| KIE20A | 1.66        | 4.940242           | 4.673723     | 5.74E-06      | 0.012717     | —         |
| MAFB   | 1.64        | 6.815799           | 4.437113     | 1.58E-05      | 0.015559     | 24        |
| EB13   | 1.64        | 5.41593            | 4.517058     | 1.12E-05      | 0.013575     | 24        |
| MELK   | 1.63        | 6.413027           | 4.141523     | 5.27E-05      | 0.034718     | —         |
| TOP2A  | 1.58        | 4.997892           | 4.045113     | 7.72E-05      | 0.040893     | 24        |
| NUSAPI | 1.54        | 6.627514           | 3.928091     | 9.35E-05      | 0.049772     | 24        |
| RGL1   | 1.52        | 7.288761           | 4.447937     | 1.51E-05      | 0.015559     | 24        |
| ARHGEF40 | −1.66    | 6.880613           | −3.994983    | 9.35E-05      | 0.043686     | —         |
| LOC254896 | −1.65   | 8.498379           | −4.452352    | 1.48E-05      | 0.015558     | —         |
| SLC46A2 | −1.61       | 5.880150           | −4.084894    | 6.60E-05      | 0.039833     | —         |
| TNFRSF10C | −1.54     | 8.678817           | −4.643272    | 6.55E-06      | 0.012717     | 24        |

**Table 3.** Functional enrichment of differentially expressed genes. BP: Biological Process; MF: Molecular Function; CC: Cellular Component.

| Functional Category | ID       | Functional Term                  | Gene Count | adjusted p-value | Fold Change | Benjamini Score |
|---------------------|----------|----------------------------------|------------|------------------|-------------|-----------------|
| BP                  | GO:0000070 | Mitotic sister chromatid segregation | 4          | 4.82E-05         | 54.83       | 0.0246          |
| BP                  | GO:0006955 | Immune response                  | 8          | 1.80E-04         | 6.51        | 0.0453          |
| BP                  | GO:0051301 | Cell division                    | 7          | 4.61E-04         | 6.85        | 0.0763          |
| BP                  | GO:0078098 | Chemokine-mediated signaling     | 4          | 0.001093         | 19.30       | 0.0776          |
| BP                  | GO:0007059 | Chromosome segregation           | 4          | 9.64E-04         | 20.15       | 0.0797          |
| BP                  | GO:007067  | Mitotic nuclear division         | 6          | 6.88E-04         | 8.29        | 0.0851          |
| BP                  | GO:0030593 | Neutrophil chemotaxis            | 4          | 8.84E-04         | 20.76       | 0.0873          |
| MF                  | GO:0008009 | Chemokine activity               | 4          | 3.63E-04         | 28.12       | 0.0467          |
| KEGG pathway        | hsa05323  | Rheumatoid arthritis             | 5          | 2.58E-04         | 15.03       | 0.0226          |
| KEGG pathway        | hsa04110  | Cell cycle                       | 5          | 9.49E-04         | 10.66       | 0.0413          |
| InterPro            | IPR001811 | Chemokine interleukin-8-like domain | 4          | 2.01E-04         | 34.33       | 0.0247          |
| InterPro            | IPR00827  | CC chemokine, conserved site      | 3          | 0.001602         | 49.35       | 0.0953          |
We identified CDC20 as one of the top upregulated genes, along with LCN2, and CD24, similar to the findings of Dong et al.25, which studied development of trauma-induced sepsis in patients. However, our study population was much more diverse (Table 2 and Supplementary Dataset 1). The most significant upregulated gene identified was DDIT4 (DNA damage-inducible transcript 4-like). PERSEVERE-XP study had also identified DDIT4 gene directly related to TP537. DDIT4 (REDD1) is increased in septic shock, can negatively regulate mTORC1 activity and plays an important role in energy homeostasis26. We found CCL3 as the second-most significant upregulated chemokine, a fundamental component of acute-phase response to endotoxin in humans and regulation of leukocyte activation and trafficking27. Elevated levels of CCL3 have been detected within the first 24 hours of sepsis, suggesting its unique role in innate immune function28,29. Further studies are needed to understand the mechanisms of these identified genes in septic shock development. On the other hand, TNFRSF10C, a downregulated gene has been shown to play an essential role in sepsis immune response30.

The set of genes identified is then examined for over-representation of specific functions or pathways. Septic shock survivors were distinguished from non-survivors by differential expression of genes involved in the immune response, chemokine-mediated signaling, neutrophil chemotaxis, and chemokine activity. Sepsis impacts the immune responses by directly altering life span, production, and function of effector cells responsible for homeostasis31. We identified the immune response (GO:0006955) term from DAVID analysis and there has also been evidence that understanding the immune response to sepsis provides opportunities to develop effective treatment strategies32.

Chemokines play a critical role in sepsis and septic shock development, and molecules that block chemokine and chemokine receptor activity may prove to be useful in the identification of sepsis33. Our differentially expressed genes mapped to chemokine-mediated signaling (GO:0070098), chemokine activity (GO:0008009) molecular function, chemokine interleukin-8 like domain (IPR001811), and chemokine conserved site (IPR000827).

Sepsis and rheumatoid arthritis (RA) have been known to be associated for over 50 years34. RA is shown to be a risk factor in sepsis patients and sepsis infection could trigger RA35. We identified RA KEGG pathway (hsa:05323) using our differentially expressed gene set to be statistically significant (Table 3). We analyzed the literature to look for other underlying genetic factors responsible for septic shock. Five (CCL3, CDC20, TNFRSF10C, EBI3, TOP2A) of the 53 identified genes were already shown to be involved in sepsis, and the remaining 48 genes from our list were not shown to be involved in sepsis. These genes may contribute to the septic shock response. Table 1 and the Discussion section above explains it in further detail. The SLC46A2 is downregulated in the septic shock non-survivor group (Fig. 1) and have been shown to be involved in T-cell homeostasis (GO:0043029). On the other hand, the LCN2 was identified as the upregulated gene and recently proposed as a biomarker involved in acute kidney injury (AKI)36. Overall, 22.6% (41/181) patients had AKI, whereas, in the non-survivors, there were 70% (19/27) patients who had AKI (see Supplementary Dataset 1 for more details).

Gene expression changes shown in our results are based on the peripheral blood cells and may not be extrapolated as occurring at the organ or tissue level37,38. Therefore, extra care must be taken while generalizing host immune responses or chemokine activities in septic shock patients. Besides, variations in gene expression profiles of survivors and non-survivors of septic shock patients could be due to other unexplored confounding factors (such as patient demographics) rather than sepsis-related biology38. On the other hand, the blood-based
biomarkers have the advantage of being minimally-invasive. Therefore, in combination with other clinical phenotypic data, these minimally-invasive biomarkers may enable rapid recognition of poor long-term outcomes. Large cohorts replication studies and network analysis studies are needed to gain insights into relationships between these biomarkers and the survival/non-survival of cohorts. To avoid the selection bias, analysis must be expanded to other independent data sets.

This work can be expanded by experimentally validating the identified blood-based biomarkers and developing robust machine learning methods to build a septic shock prediction models using different omics data from diversified patient cohorts.

Materials and Methods

Sepsis and septic shock. Bacteremia or another pathogenic infection triggers a serious inflammatory response called sepsis, which typically includes an increased number of white blood cells, rapid heart rate and breathing rate, and fever. The response also affects many major organ failures. Sepsis that results in poor perfusion to end organs resulting in multiple organ failure is defined as a septic shock.

Data collection. Expression microarray data was collected from the NCBI Gene Expression Omnibus repository. The dataset contains gene expression profiles of peripheral blood samples from 181 septic shock patients including 154 survivors and 27 non-survivors who were admitted to the pediatric intensive care unit (PICU) within the first 24 hours. For all 181 samples, blood was drawn on day 1 of the PICU stay. GEO accession number for data used in the study is GSE66099. Data was collected from the Affymetrix Human Genome HG-U133_Plus_2 (GPL570 platform).

Normalization and background correction. The R Affy module was used to remove technical variations and background noise. The Quantile Normalization Method was used to normalize data and background correction was performed using the Robust Multi-Average parameter method.

Probe to gene mapping. Affymetrix probes were mapped to the genes using information provided in the Affymetrix database (hgu133plus2.db). We used average expression values when multiple probes mapped to the same gene.

Identification of differentially expressed genes. Differentially Expressed Genes (upregulated and downregulated genes) were identified using R limma package with a Benjamini-Hochberg (BH) correction method and adjusted p-value of <0.05.

Functional analysis. We used DAVID for functional enrichment analysis of the DEGs from samples of septic shock survivors or non-survivors. Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) were identified from the Gene Ontology database. For the GO functional groups, KEGG pathways, and InterPro functional terms returned from DAVID functional analysis, we considered an adjusted p-value threshold of ≤0.05 and gene count of 3 or more from this study.

Statistical analysis. R programming language was used for downloading the Affymetrix data and gene mapping using R Affy and Bioconductor package. A Fisher-exact test was performed for determining statistical significance among gene ontology terms and functional classes. Benjamini Hochberg multiple test correction method was used for calculating the differentially expressed genes. Heatmap of differentially expressed genes between survivor and non-survivor groups for top selected genes were plotted using the complexHeatmap function in R programming. Other variables (pathogen, gram-positive, immunosuppression, gender status, and infection source) were added to the heatmap for annotation.

Data Availability

R scripts other related files used for data preprocessing, normalization and differential gene expression analysis are available from https://github.com/akram-mohammed/septic_shock_degs. Datasets generated and analyzed during the study are available upon request.

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**Author Contributions**
R.K. and A.M. conceived and designed the study, developed the method and performed the analysis. Y.C. and V.R.M. contributed to the analysis. All authors wrote and proofread the manuscript.

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