J Surg Med. 2022;6(12):932-938.
DOI: 10.28982/josam.7461

Journal of Surgery and Medicine
e-ISSN: 2602-2079

Identification of novel potential molecular targets associated with pediatric septic shock by integrated bioinformatics analysis and validation of *in vitro* septic shock model

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Ethics Committee Approval
The study is a bioinformatics study and raw data was used. The database for which we use the data, Gene Expression Omnibus (GEO), is a public database and therefore ethical approval is not required.

Conflict of Interest
No conflict of interest was declared by the authors.

Financial Disclosure
The authors declared that this study has received no financial support.

Published
2022 December 1

Abstract

Background/Aim: Sepsis is a major cause of morbidity, mortality, and healthcare utilization among children all over the world. Sepsis, characterized as life-threatening organ failure, results from a dysregulated host response to infection. When combined with critically low blood pressure, it causes septic shock, resulting in high mortality rates. The aim of this study was to perform a bioinformatic analysis of gene expression profiles to predict septic shock risk.

Methods: Four datasets related to pediatric septic shock were retrieved from the Gene Expression Omnibus (GEO) database for a total of 240 patients and 83 controls. GEO2R tools based on R were used to find differentially expressed genes (DEGs). The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to examine the functional enrichment of DEGs. STRING was used to create a protein–protein interaction (PPI) network. After separately analyzing the four datasets, commonly affected genes were removed using the Venny program. Finally, human umbilical vein endothelial cells (HUVECs) were stimulated with supernatants of lipopolysaccharide (LPS)-stimulated RAW267.4 macrophage cells and expression of selected genes was confirmed by real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) and used to construct an *in vitro* septic shock model.

Results: Seven-hundred seventy-one common differentially expressed genes in the four groups were found. Of these, 433 genes showed increased expression, while 338 had reduced expression. In the DAVID analysis results, DEGs up-regulated according to gene ontology results were enriched in the regulation of innate and adaptive immune responses, complement receptor-mediated signaling, and cytokine secretion processes. Down-regulated DEGs were significantly enriched in the regulation of immune response, T-cell activation, antigen processing, and presentation and integral component of plasma membrane processes. According to The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), Cystoscape Molecular Complex Detection (MCODE), nine down-regulated genes in the center of the PPI network, ZAP70, ITK, LAT, PRKCQ, LCK, IL2RB, FYN, CD8A, CD247 and four up-regulated genes, MMP9, TIMP1, LCN2, HGF, were associated with septic shock. Expressions of FYN and MMP9 genes in the *in vitro* septic shock model were consistent with the bioinformatic results.

Conclusion: Comparative bioinformatics analysis of data from four different septic shock studies was performed. As a result, molecular processes and important signal networks and 13 genes that we think will play a role in the development and risk prediction of septic shock are proposed.

Keywords: Pediatric sepsis, Septic shock, Bioinformatics, Hub gene, Biomarker, Differentially expressed genes

How to cite: Gürel AK, Gürel S. Identification of novel potential molecular targets associated with pediatric septic shock by integrated bioinformatics analysis and validation of *in vitro* septic shock model. J Surg Med. 2022;6(12):932-938.

Introduction

Sepsis is a major cause of morbidity, mortality, and healthcare utilization among children all over the world. Globally, 22 pediatric sepsis cases occur per 100,000 people annually, and 2,202 neonatal sepsis cases occur per 100,000 live births. This number is equivalent to 1.2 million pediatric cases annually [1]. Sepsis is defined as a life-threatening disease that causes septic shock and organ dysfunction by producing a systemic inflammatory response. Despite breakthroughs in antibiotic treatment, immunotherapy, and resuscitative treatments, sepsis remains the leading cause of mortality in critical care units [2, 3]. Despite many studies in the field of sepsis treatment in past years, studies directly targeting sepsis, those addressing treatments to ensure amelioration, and preclinical studies have not been promising. Generally, the current strategy chosen for sepsis treatment involves targeting the infective pathogen in sepsis rather than patient response. Therapeutic choices for sepsis are limited because the responsible pathogenic mechanisms are still not fully understood [4]. Septic shock is a homogeneous disease without a single cause but rather enters a broad heterogeneous disease classification that includes subclasses of several diseases. The subclass definitions of septic shock are clinically significant as they may have clear effects for design of potentially targeted treatments [5]. Studies were performed concerning the subclassifications of patients with septic shock using biological markers found in serum. Considering the complexity of septic shock, definitions of new biomarkers in biological terms is very important [6].

Microarray analysis is a broad-scope technique that can analyze all identified transcripts concurrently instead of analyzing single gene expression. The basic aim of experiments involving gene expression is to determine genes displaying different behaviors under different conditions and to identify a reliable measurement for these differentially expressed genes. Genome scale association studies use the integration method to discover new gene sets [7]. Consolidation analysis, which collects information from different studies about the same topic to reveal guiding results about genes and pathways commonly targeted in disease, has more statistical power than analyses based on a single study. Additionally, this technique is used to reveal disease subtypes, predict survival, and discover biomarkers and treatment targets in gene expression studies [8, 9].

To date, several studies about determining molecular signatures in pediatric septic shock patients have been performed. Within the scope of these studies, research has been performed to determine molecular signatures with many patients and controls by analyzing studies performed involving septic shock patients and healthy controls. Early diagnosis and accurate prognostic prediction of septic shock are very important in terms of successful disease treatment. In this context, gene expression is considered an important tool to fill a gap in the complicated network of septic shock treatment [8, 10]. The aim of this study was to determine the common role of differentially expressed genes (DEGs) in pediatric sepsis, to provide specific information for clinical sepsis treatment in children, and to research potential therapeutic targets and biomarkers during sepsis development using comprehensive bioinformatics analyses from four different datasets.

Materials and methods

Bioinformatic analysis of microarray data:

In our research, studies including all pediatric septic shock expression profiles from the publicly available functional genomic Gene Express (GEO) database were queried and four datasets (GSE26440, GSE9692, GSE26378, GSE8121) were used (http://www.ncbi.nlm.nih.gov/geo). The sample numbers in each dataset are listed: (1) GSE26440 includes 98 cases and 32 controls, (2) GSE9692 includes 30 cases and 15 controls, (3) GSE26378 includes 82 cases and 21 controls, and (4) GSE8121 includes 30 cases and 15 controls. All profiles were based on GPL570 Affymetrix Human Genome U133 Plus 2.0 Array. For bioinformatics analysis of studies performed with blood samples taken from pediatric septic shock patients and control groups, the R-based GEO2R software (http://www.ncbi.nlm.nih.gov/geo/geo2r) was used. To ensure a balance between the discovery of statistically significant genes and adjusted P value calculations to obtain |log2 (Fold Chance (FC) |, GEO2R was used to calculate adjusted P values (adjP) and Benjamini–Hochberg false discovery rates. Genes with $P < 0.001$ and $|\log2FC| \geq 1$ were accepted as DEGs. Later, overlapping results were created from different gene lists using a Venn diagram (https://bioinfogp.cnb.csic.es/tools/venny/).

Identification of DEGs: The Database for Annotation, Visualization and Integrated Discovery (DAVID) is a web-based gene function enrichment analysis software and is the main bioinformatics tool for analyzing the biological processes associated with DEGs. Statistical significance for DEGs and gene ontologies (GO) were found using DAVID. GO is a large and widely used database for categorizing gene functions into biological processes (BP), molecular functions (MF), and cell components (CC). The Kyoto Encyclopedia of Genes and Genomes ([KEGG]http://www.kegg.jp/) is a genome encyclopedia combining genomic information with more high-grade functional information to identify enriched biological paths to a significant degree. Significant genes and pathways (increasing or decreasing) with $p < 0.05$ were analyzed using gene enrichment and KEGG pathway analyses.

Protein–protein interaction analysis and Hub Gene Identification: STRING (http://string-db.org,version 11.0) is an online database providing information about protein–protein interactions (PPIs) via an analysis of functional networks between two or more proteins. The highest reliability point of 0.9 was used for interaction networks and nodes without interactions were removed. Cytoscape (version 3.8.2) software is an open access bioinformatics platform created to visualize PPI networks. Network analysis was performed by applying the Cystoscape Molecular Complex Detection (MCODE, version 1.5.1), an application within Cytoscape.

Validation of microarray results

Cell culture

In our study, two cell lines, macrophage RAW267.4 cells and human umbilical veil endothelial cells (HUVECs) were used, and the cells were obtained from the American Type

Culture Collection (ATCC) cell bank. RAW267.4 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FBS (Hyclone) and 100 U/mL of penicillin and streptomycin (Gibco). HUVECs were grown in F12 medium (Gibco), which contained 15% FBS, antibiotics, and 30 g/mL EGFS (Sigma). Cell counts were performed on a thoma slide using trypan blue to seed 1x105 viable cells in each well before seeding into plates.

Cell viability assay: The cell viability of RAW264.7 cells after stimulation was determined using the tetrazolium (MTT) test. MTT dye was dissolved in RPMI 1640 medium to a final concentration of 2 mg/ml. After discarding the medium on the cells, 20 µl of MTT solution and 100 µl of medium were added to each well in a 96-well plate and incubated at 37 °C for 1 h in a CO_2 incubator. Absorbance was measured at 550 and 690 nm using a spectrophotometer.

***In vitro* septic shock cell model:** The septic shock cell model consisted of two steps. In the first step, the Raw267.4 cells were seeded in 6- and 96-well plates and stimulated with 10 µg/ml lipopolysaccharide (LPS) based on studies in the literature. The cells were incubated for 4, 8, and 24 h, and the culture supernatant was collected at specified hours (containing various factors secreted by cells in response to stimulation). In the second step, HUVECs were seeded in 96- and 6-well plates at a concentration of 1 x 10^5 cells and incubated for one night.HUVEC medium was then cultured with the supernatants of LPS-stimulated macrophage cells and incubated for 0, 12, 24, and 48 h. The cells were isolated and stored at –80 ºC.

Isolation of RNA and qRT-PCR

In the study, the expression of MMP9 and FYN genes was quantitatively analyzed using the real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) method. Trizol was used to extract total RNA from cells (Invitrogen). Reverse transcriptase was used to synthesize complementary DNA (Roche). qRT-PCR experiments were carried out using SYBR Green PCR Master Mix (Roche). The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an endogenous control for normalizing mRNA expression levels. Matrix metallopeptidase 9 (MMP9) with upregulated expression and FYN Pro-to-Oncogene, Src Family Tyrosine Kinase (FYN) with downregulated expression were chosen. Primers were used as in the references provided [11–13].

Statistical analysis

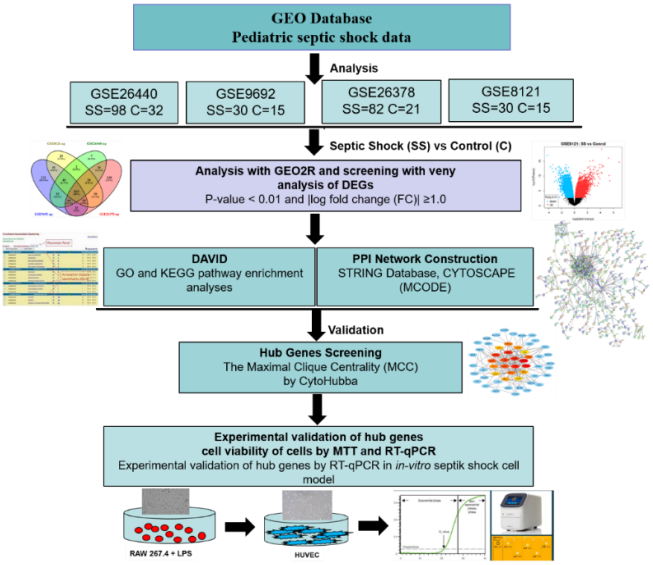
All results were presented as the mean standard error (SE) of three separate experiments. For statistical analysis, a one-way analysis of variance (ANOVA) test or an unpaired t-test was used. All statistical tests were two-tailed with a $P < 0.001$ threshold.

Results

Pre-processing of microarray data

The gene expression data for GSE26440, GSE9692, GSE26378, and GSE8121 were downloaded from the GEO general functional genomic database with the Affymetrix human genome U133 Plus 2.0 platform. Genes with *P*-value < 0.001 and |log2FC| ≥ 1 were chosen. A large group was created, with n = 240 patients and n = 83 controls (Figure 1).

Figure 1: Flow diagram of the bioinformatics analyses performed in this study (SS: Septic Shock, C: Control).



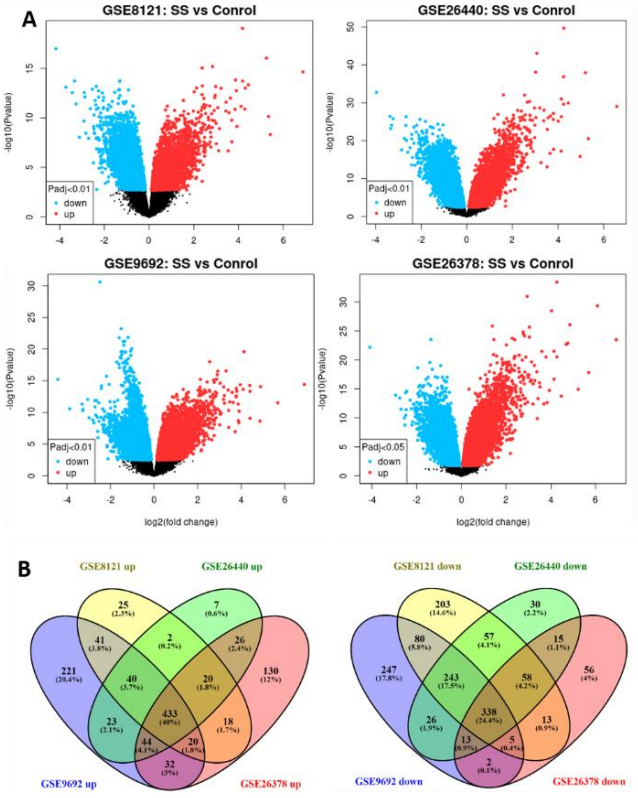
Identification of DEGs

In GSE26440 data, a total of 1375 genes had changed, with 595 genes showing increased expression and 780 genes showing decreased expression. For GSE9692 data, 1808 genes were differentially expressed, 854 of which were upregulated and 954 of which were downregulated. For GSE26378 data, the expression of 1223 genes had changed, with 723 upregulated genes and 500 downregulated genes. For GSE8121 data, expression differed for 1596 genes, with 599 upregulated and 997 downregulated genes (Table 1 and Figure 2A–B).

Table 1: Genes count with differentially expressed after bioinformatics analysis for comparison of between groups.

GEO Access Number	Number of DEGs	Upregulated gene number	Downregulated gene number
GSE26440	1375	595	780
GSE9692	1808	854	954
GSE26378	1223	723	500
GSE8121	1596	599	997

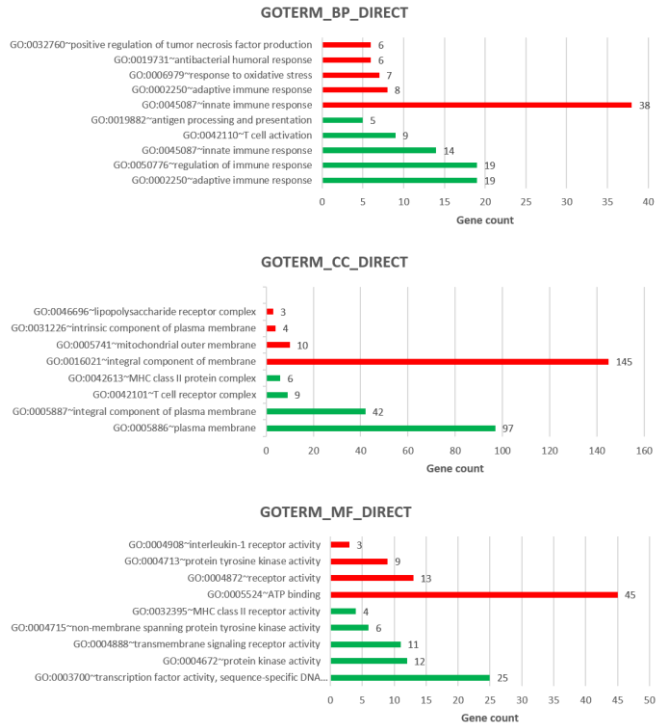
Figure 2: Venn diagram and volcano diagram of DEGs. A: Volcano diagram of 4 datasets. B: DEGs were selected with a |log2FC ≥ 1| and $P < 0.001$ among above 4 mRNA expression profiling sets datasets. The 4 datasets showed an overlap of 771 genes (433 upregulated, 338 downregulated).



After separately analyzing the four datasets, the commonly affected genes were identified using the Venny program. Seven-hundred seventy-one genes changed among the four datasets. Of these, 433 gene showed an increase in expression, and 338 had a reduction in reduced expression ($P < 0.001$).

GO Function and KEGG pathway analysis with DAVID: To better understand the functions and mechanisms of the DEGs identified after analysis of microarray data, GO and KEGG pathway analyses were completed for genes with increasing or decreasing expression levels. In GO analyses, clusters with enrichment score above 1.3 were assessed as significant clusters. According to this assessment, enrichment was observed for 27 clusters with up-regulated expression and 16 clusters with down-regulated expression based on the GO analysis of genes with common changes in the four studies. According to GO for DAVID analysis results, DEGs were collected from CC, especially in plasma membrane (97 genes) and integral membrane components (187 genes). Significant enrichment for BP in innate and adaptive immune responses and regulation of immune response (84 genes) processes and for MF in transcription factor activity, sequence-specific DNA binding (25 genes), and ATP binding (45 genes) processes was found. The GO functional enrichment analysis of up-regulated and down-regulated DEGs is shown in Figure 3 ($P < 0.001$).

Figure 3: GO functional enrichment analysis of downregulated DEGs ($P < 0.001$). The red and green colors represent upregulation and downregulation, respectively (BP indicates biological process; CC, cellular component; MF, molecular function).



For the common differentiated genes obtained from the four datasets, KEGG pathway analysis found that upregulated DEGs were basically related to metabolic pathways, neutrophil extracellular trap formation, osteoclast differentiation, transcriptional mis-regulation in cancer, and complement and coagulation cascades pathways. Additionally, the down-regulated DEGs revealed significant degrees of enrichment in a total of 10 pathways led by T-helper cells (Th) 1, 2, and 17 cell differentiation, hematopoietic cell lineage, T-cell receptor signaling pathway, and cytokine–cytokine receptor interaction pathways ($P < 0.001$) as shown in Table 2.

Table 2: KEGG pathway analysis of DEGs associated with septic shock using DAVID.

A. KEGG Pathways of Upregulated Genes

Term	Count	*P*-value	Genes
hsa01100:Metabolic pathways	55	0.005	CDA, GALNT14, PYGL, ENO1, OPLAH, HK3, SPTLC2, IMPA2, NAMPT, CA4, MAN1A1, UPP1, GYG1, ENTPD1, G6PD, DGAT2, ACSL1, ARG1, ACSL4, PGD, CHIT1, PKM, ACOX1, B3GNT5, ST6GALNAC3, BCAT1, B4GALT5, PFKFB2, PFKFB3, MGST1, ADCY3, PLD1, CYP19A1, GNS, PGS1, UGCG, FUT7, ALOX5, GPAT3, ATP6V1C1, OLAH, MGAM, GK, GALNT2, GSR, MBOAT2, BST1, VNN1, DHRS9, TBXAS1, ACER3, LPCAT2, ALPL, HPSE, GCLM
hsa04613:Neutrophil extracellular trap formation	15	0.001	CR1, ITGAM, SIGLEC9, AQP9, NCF4, C5AR1, FPR1, FPR2, MAPK14, MPO, TLR8, PADI4, TLR4, ELANE, TLR2
hsa04380:Osteoclast differentiation	14	0.001	LILRA6, SPI1, IL1R1, IFNGR1, NCF4, LILRB3, MAPK14, LILRA3, LILRA5, FOSL2, OSCAR, SOCS3, SIRPA, MAP2K6
hsa05202:Transcriptional misregulation in cancer	13	0.003	SPI1, ITGAM, BCL2A1, GADD45A, DEFA4, IL1R2, LMO2, MPO, MMP9, CCNA1, BCL6, CD14, ELANE
hsa04610:Complement and coagulation cascades	12	0.001	C1QB, C1QA, SERPINA1, CR1, ITGAM, SERPINB2, C5AR1, C3AR1, CD59, VSIG4, CD55, F5
hsa04621:NOD-like receptor signaling pathway	11	0.018	AIM2, DEFA4, NAMPT, PRKCD, CARD6, CARD16, NLRC4, TXN, MAPK14, TLR4, NAIP
hsa05150:Staphylococcus aureus infection	9	0.002	C1QB, C1QA, ITGAM, DEFA4, C5AR1, FPR1, C3AR1, FPR2, FCAR
hsa04640:Hematopoietic cell lineage	9	0.003	CSF3R, CR1, ITGAM, IL1R1, IL1R2, CD59, CD14, CSF2RA, CD55
hsa04620:Toll-like receptor signaling pathway	8	0.016	LY96, TLR8, CD14, MAPK14, TLR5, TLR4, MAP2K6, TLR2
hsa04064:NF-kappa B signaling pathway	8	0.016	BCL2A1, IL1R1, GADD45A, TRIM25, LY96, CD14, CFLAR, TLR4

B. KEGG Pathways of Downregulated Genes

Term	Count	*P*-value	Genes
hsa04658:Th1 and Th2 cell differentiation	19	0.001	MAML2, NFATC2, CD3G, GATA3, CD3E, RUNX3, CD3D, ZAP70, HLA-DMB, LCK, IL2RB, HLA-DPB1, STAT4, PRKCQ, CD247, HLA-DOA, HLA-DOB, LAT, HLA-DPA1
hsa04659:Th17 cell differentiation	18	0.001	NFATC2, RORA, CD3G, GATA3, CD3E, CD3D, IL27RA, ZAP70, HLA-DMB, LCK, IL2RB, HLA-DPB1, PRKCQ, CD247, HLA-DOA, HLA-DOB, LAT, HLA-DPA1
hsa04640:Hematopoietic cell lineage	17	0.001	MME, ITGA4, FLT3LG, CD3G, CD1C, CD3E, CD3D, CD2, HLA-DMB, CD8A, HLA-DPB1, ITGA6, HLA-DOA, IL7R, MS4A1, HLA-DOB, HLA-DPA1
hsa04660:T cell receptor signaling pathway	14	0.001	ITK, NFATC2, CD3G, CD3E, RASGRP1, CD3D, ZAP70, LCK, CD8A, CD28, FYN, PRKCQ, CD247, LAT
hsa04060:Cytokine-cytokine receptor interaction	13	0.002	CX3CR1, IL27RA, CCL5, IL2RB, TNFRSF17, XCL1, CD27, ACKR3, TNFRSF25, CCR7, CCR6, IL7R, CCR3
hsa04650:Natural killer cell mediated cytotoxicity	11	0.001	ZAP70, LCK, KLRC3, SH2D1A, SH2D1B, PRF1, NFATC2, KLRD1, FYN, CD247, LAT
hsa04514:Cell adhesion molecules	11	0.001	CD2, HLA-DMB, CD6, ITGA4, CD8A, HLA-DPB1, CD28, ITGA6, HLA-DOA, HLA-DOB, HLA-DPA1
hsa04064:NF-kappa B signaling pathway	9	0.001	CYLD, ZAP70, LCK, TRAF5, BLNK, BCL2, PRKCQ, ATM, LAT
hsa04612:Antigen processing and presentation	9	0.001	CD74, HLA-DMB, CD8A, KLRC3, HLA-DPB1, KLRD1, HLA-DOA, HLA-DOB, HLA-DPA1
hsa04062:Chemokine signaling pathway	9	0.011	CX3CR1, TIAM1, ITK, CCL5, XCL1, CCR7, CCR6, PRKACB, CCR3

Protein-–protein interaction (PPI) analysis

To research the molecular pathogenesis of septic shock, PPIs of the common differentiating genes were researched using gene expression profiling data with multiple bioinformatics methods, including gene enrichment analysis and PPI analysis. The STRING online database was used to create the PPI network using a total of 771 common DEGs (433 up-regulated, 338 down-regulated), which was analyzed by choosing the high reliability points of 0.9 for the interaction network, and then transferred to Cytoscape. The PPI network for the common down-regulated DEGs was visualized by Cytoscape and identified by the MCODE macro, an important module in Cytoscape and MCODE score >3 was chosen. Analysis of down-regulated genes found 149 edges and 64 key nodes. Three clusters with high MCODE score were investigated and hub genes were identified as ZAP70, ITK, LAT, PRKCQ, LCK, IL2RB, FYN, CD8A, CD247, CD3E, CD28, CD3D, CD3G, CD74, HLA-DPA1, HLA-DOA, HLA-DOB, HLA-DMB, HLA-DPB1, CAMK4, MEF2C, and TRAC. For up-regulated genes, 134 edges and 133 key nodes were identified. When MCODE classification is performed, MMP9, TIMP1, LCN2, HGF, PFKFB4, PFKFB2, PFKFB3, FPR1, FPR2, IL1R2, ANXA1, IL1RN, IRAK3, IL1R1, ENO1, PKM, and G6PD emerged as

hub genes (Table 3). Genes included in the first cluster with highest MCODE score for down-regulated genes were mostly associated with the T-cell receptor signaling pathways, and apart from this, the primary immunodeficiency, natural killer cell-mediated cytotoxicity, hematopoietic cell lineage, and nuclear factor kappa beta (NF-kappa B) signaling pathways were involved (Table 3 and Figure 4).

Table 3: The top significant modules in PPI networks were identified using MCODE.

Genes	Cluster	Score	Nodes	Edges	Node IDs
Downregulated	1	11	13	68	ZAP70, ITK, LAT, PRKCQ, LCK, IL2RB, FYN, CD8A, CD247, CD3E, CD28, CD3D, CD3G
	2	6	6	15	CD74, HLA-DPA1, HLA-DOA, HLA-DOB, HLA-DMB, HLA-DPB1
	3	3	3	3	CAMK4, MEF2C, TRAC
Upregulated	1	3	4	5	MMP9, TIMP1, LCN2, HGF
	2	3	3	3	PFKFB4, PFKFB2, PFKFB3
	3	3	7	9	FPR1, FPR2, IL1R2, ANXA1, IL1RN, IRAK3, IL1R1
	4	3	3	3	ENO1, PKM, G6PD
	5	3	3	3	C1QA, C1QB, VSIG4
	6	3	3	3	PRKCD, HCK, FGR

Figure 4: PPI network's MCODE components identified genes associated with septic shock. Modules discovered through the MCODE algorithm. A: 6 clusters obtained with upregulated genes. B: 3 clusters obtained with downregulated genes.

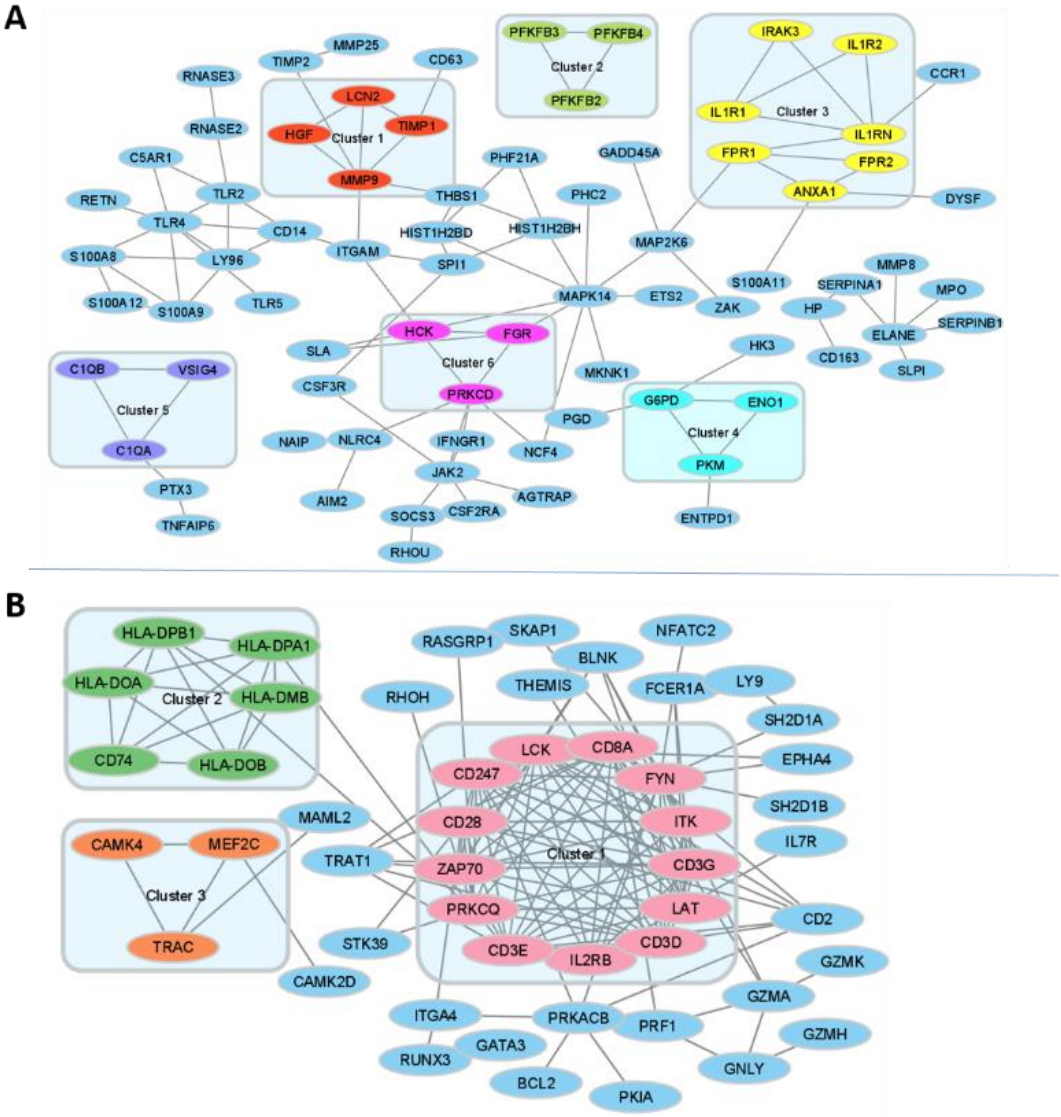
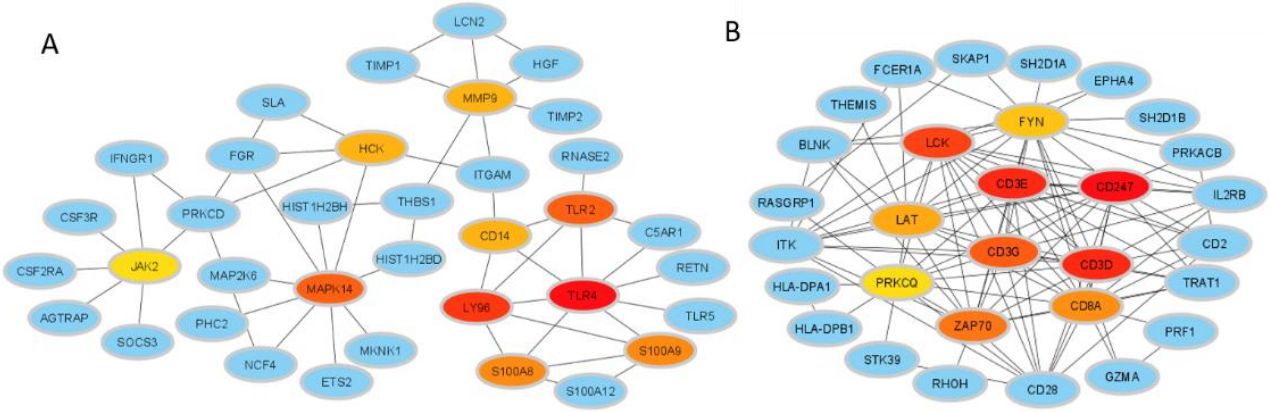


Figure 5: Visualization of the PPI network and the candidate hub genes. Identification of the hub genes from the PPI network using MCC algorithm. A: PPI network of the upregulated genes B: PPI network of the downregulated genes. The red nodes represent genes with a high MCC sores, while the yellow nodes represent genes with a low MCC score.



Identification of the Hub Gene

CytoHubba (part of Cytoscape software)was used to identify significant hub genes among the resulting DEGs. The top 10 genes in the PPI network were chosen using the MCC method. A of connectivity in the PPI network was then used to evaluate the top 10 genes for selected up- and down-regulated genes. Accordingly, down-regulated genes CD247, CD3E, CD3D, LCK, CD3G, ZAP70, CD8A, LAT, FYN, and PRKCQ and upregulated genes TLR4, LY96, TLR2, MAPK14, S100A9, S100A8, MMP9, CD14, HCK, IL1RN were prominent as hub genes. It is noteworthy that the genes with the highest degree consisted of down-regulated genes (Figure 5A and B).

Validation of Hub Gene Expression

Cell viability and qRT-PCR validation results

After using an integrated bioinformatics approach, the resulting key genes were validated in a well-established model of septic shock. qRT-PCR analysis was performed for the relative fold change in expression. We correlated qRT-PCR analysis with our findings from the bioinformatics analysis and validation study. In the first stage of the study, RAW267.4 was stimulated with 10 µg/mL LPS. The same dose was chosen based on results from the literature, and highest stimulation was observed in the 24th hour. As shown in Figure 5A, stimulation of RAW267.4 with LPS resulted in a decrease in cell viability at 24 h ($p < 0.05$). In our study, the relative cell viability began to decline in the 24th hour based on MTT results, and cell supernatant from this time was used. In the second stage, HUVEC cells were seeded at 1 x 105 cells/1 ml concentration on 96-well plates and incubated for one night. Later the 24-h culture supernatants collected from macrophages were stimulated for 0, 12, 24, and 48 h. From the 48th hour, cells were observed to have a significant degree of numerical reduction ($P < 0.001$) as shown in Figure 6A and B.

In studies performed with the aim of mimicking a septic shock model *in vitro*, samples were collected from HUVEC and treated for 0, 12, 24, and 48 h very hour with culture supernatant from the stimulated macrophages. After PCR was performed with the aim of optimizing primers designed for *MMP9, FYN*, and *GAPDH*, the resulting cDNA was subject to a qRT-PCR analysis.. The MMP9 cell model was up-regulated at 0 h, in other words, initially in the control group without application. From the 12th hour, gene expression began to decline, and this down-regulation continued until the 48th hour. The MMP9 microarray fold ratio was 6.9 with an increase in the qRT-PCR fold ratio of 3.5-fold.. As cell models with changes occurring in the same direction as array results are usable, this verifies that this is a model that can be used for drug trials mimicking septic shock or for confirming microarray studies. In the FYN cell model, down-regulation occurred starting at 0 h. The microarray fold ratio was –1.4, with qRT-PCR fold ratio of –2.4 at 0 h, 0.45 at the 12th hour, –1.6 at the 24th hour, and –1.5 at the 48th hour. Our results occurred in the same direction as the array results (Figure 7).

Figure 6: Cell viability assay results A: The MTT cell proliferation test was performed with the aim of checking cell death. RAW 264.7 cells were stimulated with 10 µg/mL LPS at 4, 8, and 24 hours. B: Delivery of supernatant from stimulated RAW 267.4 cells to HUVECs and cell viability at different time points (0, 12, 24, and 48 hours).

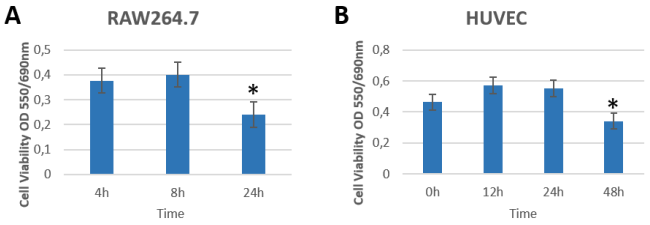
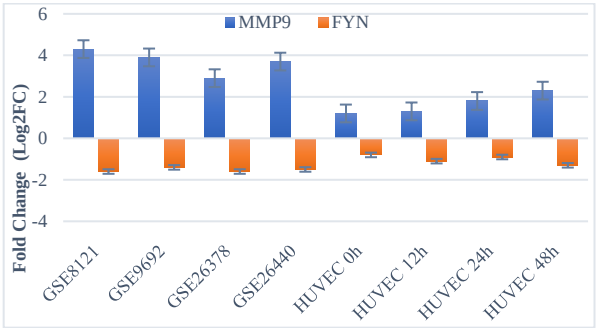


Figure 7: Relative expression of hub genes in an *in vitro* septic shock model. Quantitative real-time PCR results for validation.



Discussion

Sepsis and septic shock are leading causes of morbidity and mortality in intensive care units. Although the death rates from septic shock have improved in recent years, they still remain very high [14]. Many genes revealed in studies in recent years were reported to be associated with septic shock mortality [15]. It is difficult to determine the most important candidate genes and pathways for prognosis at present due to the complicated background of septic shock. Transcriptome studies revealed differences in gene expression profiles and responses throughout the genome [16]. With the growth of high efficiency transcriptomic data, it is possible to perform integrative analysis with multiple datasets to discover reliable candidates for prognosis and treatment. For this reason, we performed integrated analysis increasing the patient and control numbers by using four different datasets to determine potential transcriptomic markers for prognosis in pediatric septic shock and targeted the common genes and pathways playing roles in septic shock. The present study found a total of 771 differentially expressed genes (433 up-regulated, 338 down-regulated) in common in the four datasets for septic shock patients.

As a result of analysis, FYN and CD247 among the hub genes were found to be positively associated with survival in sepsis in studies. Additionally, meta analysis results showed that FYN may be beneficial for prognosis in patients and that CD247 may differentiate patients with sepsis and systemic inflammatory response syndrome. RNA sequencing using a mouse septic shock model showed that CD247 and FYN expression levels were low in this model [17]. Similar to our study, studies of these genes found similarly low expression levels.

Another study of pediatric and adult sepsis and septic shock patients identified MAPK14, FGR, RHOG, LAT, PRKACB, UBE2Q2, ITK, IL2RB, and CD247 as controls of hub genes. They also found stated irregularities between sepsis patients and septic shock patients, and also that especially expression of MAPK14, FGR and CD247 was regulated by the

methylation pathway. They stated that these findings were important to identify potential diagnostic genes associated with sepsis development and the inflammatory and metabolic response mechanisms [18].

Network analysis results found adaptive immunity was pronounced in sepsis, and a tendency to form isolated clusters with genes including CD247, CD8A, ITK, LAT and LCK was at the forefront [19].

Matrix metalloproteinases (MMPs) and metalloproteinase tissue inhibitors (TIMPs) may be promising biomarkers for prognosis during sepsis development. Hoffman et al. observed an association between mortality in septic patients and high MMP9, TIMP2, and TIMP1 plasma levels and did not find a difference in MMP while showing significantly high TIMP1 levels in those who survived compared to those who did not.. Another study found low MMP9 levels and lower MMP9/TIMP1 ratio in mortally ill septic patients [20]. In our study, MMP9 and TIMP1 expression levels were up-regulated in the septic shock group. Lipocalin-2 (LCN2) belongs to an evolutionarily preserved family comprising more than 20 members characterized by the capability of binding and transporting small hydrophobic molecules. Lipocalins are known to play a role in inflammatory diseases and cancer. Although it was determined to be a biomarker for cancer, no correlation with sepsis was found in our study. In our study, the LCN2 gene expression was high. It was first identified in cytoplasmic granules of human neutrophils [21]. It acts as an acute phase protein, and expression is induced by pro-inflammatory stimulation. The protein is included in the innate immune response. LCN2 and MMP9 genes are included among hub genes. A study of stomach cancer showed that MMP9 activity was up-regulated by LCN2, and both LCN2 and MMP9 were controlled by the NFκB pathway resulting in proliferation and invasion [21, 22]. In our results, the expression of both genes was up-regulated, leading to the notion that they are involved in inflammation and related to the NFκB pathway. This pathway can serve as a therapeutic target and will make it easier to identify other targets in sepsis and septic shock. as corrected.

The expression levels of selected genes were examined using quantitative qRT-PCR to confirm microarray data. One gene with up-regulation and one with down-regulation were chosen, and validation was performed. The qRT-PCR results showed the same correlation with those obtained from the microarray analysis. The genes have been validated in the septic shock model that we created based on the literature, but more extensive *in vitro* and *in vivo* experiments are needed to understand the pathology and investigate the gene pathways.

The PPIs and connection numbers of up-regulated and down-regulated genes are important for septic shock regulation. Strong interactions and connection power show that these genes and pathways act together. Panels created for these genes may provide a promising step forward in terms of diagnosis and treatment of septic shock. When determining these gene groups, more advanced bioinformatics analyses and functional experiments are required.

Limitations

Although our study was validated *in vitro*, it has some limitations. First, the data for analysis were downloaded from databases, and problems may arise from the differences in the experimental environments in which these studies were carried out, which may have affected the results. Second, even if molecular experiments were partially performed for validation, these preliminary results need to be performed with larger clinical samples to confirm their accuracy .

Conclusion

This study shows some genes are considered to play a role and be important for pediatric septic shock. Although many genes are known to be associated with septic shock mortality, it is of great importance to identify important candidate genes for the prognosis of the disease and the pathways in which these genes play a role. In order to evaluate the course of the disease, detecting and following the changes in terms of the amount of mRNA or mediator in the organism according to time will open new treatment avenues.. For this reason, we performed integrated analyses with multiple general microarray datasets to determine potential transcriptomic markers for prognosis of pediatric septic shock.

Acknowledgements

The authors gratefully acknowledge the NCBI-GEO for the use of raw data.

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