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Original Article

Using gene set enrichment analysis to identify new genes involved in sepsis-induced myopathy

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Abstract: Background and Aim: The aim of this study was to discover and validate novel therapeutic targets for the treatment of sepsis-induced muscle weakness using a gene set enrichment analysis approach. Methods: Publicly available sepsis-induced myopathy gene expression signatures and gene expression signatures from 107 siRNA-mediated knockdowns were analyzed using a rank-based algorithm to evaluate the similarities. This analysis provided a list of candidate human genes with similar perturbations in sepsis and siRNA knockdown that may be involved in a response to sepsis-induced myopathy. Candidate genes were validated using quantitative RT-PCR in a cecal ligation and puncture model of septic myopathy in the mouse. Results: We calculated the gene profile distances between the host responses to septic myopathy and the 107 siRNA knockdowns. Nine genes (PSIP1, TOP1, PCGF2, SRF, HIF1A, FAS, BCL11A, UPF1 and PARK7) were selected as candidate genes likely to be involved in sepsis-induced myopathy. Quantitative RT-PCR analysis in septic mice revealed that PSIP1 expression was decreased, HIF1A, FAS and UPF1 expression were increased, and the remaining genes were unchanged. Conclusion: The gene set enrichment analysis approach is an effective method to identify new genes involved in sepsis-induced myopathy. We identified PSIP1 as a factor associated with sepsis-induced muscle myopathy.

Keywords: Sepsis, myopathy, PSIP1, gene set enrichment analysis

Introduction

Sepsis is a primary contributor to morbidity and mortality in hospital intensive care units (ICUs). Despite more recent advances in treatment, the mortality rate remains high. Patients that survive sepsis are at increased risk for the development of persistent critical illness myopathy [1, 2]. The muscles of the respiratory system and the limbs are significantly affected. The weakness in these patients is not due to a lack of activity, but rather to the presence of newly acquired muscular disorders, which develop as a result of significant physiological changes occurring in the muscles without overt electrophysiological abnormalities. Results from biopsies [3] and neurophysiological studies [4] in critically ill patients suggest that neuropathy or disturbances in neuromuscular transmission may arise due to alterations in the function or structure of the muscle.

Although many factors have been associated with prolonged critical illness myopathy, sepsis was identified as a major risk factor [5]. Sepsis-induced myopathy is characterized by a decrease in muscle force-generating capacity [6, 7], muscle atrophy [8, 9], altered bioenergetics [10-12], decreased skeletal muscle excitability [13, 14], injury to the sarcolemmal membrane [15, 16], and altered calcium homeostasis [17]. The incidence of sepsis has been reported to be as high as 70-100% in patients with prolonged weakness [18-20]. Several mechanisms, such as excessive localized expression of proinflammatory cytokines [21, 22], marked increases in free-radical generation [23-25], and activation of proteolytic pathways, have been proposed to cause the sepsis-induced changes in skeletal muscle. Because of the broad spectrum of pathophysiological mechanisms, different strategies stemming from experimental evidence and data from clinical tri-

als have been developed [26], which include approaches such as nutritional interventions [27], supplement therapies [28], and physical therapy. These treatments, however, have not been correlated with improved disease prevalence or outcome. Other targets for treatment have been proposed and include antioxidants [29], testosterone derivatives [30, 31], growth hormones [32, 33], immunoglobulins (e.g., TNF-a or IL-1 receptor antagonists [34], and proteasome inhibitors [35-38]. Several genes including TNFSF1A, TNFSFIB, MYD88, and MAPK3 have been reported to be differentially expressed in sepsis or septic shock patients [39], yet knockdown of these genes has not provided a therapeutic benefit in septic patients. Many of these targets, however, have yet to be validated or show enhanced muscle function in sepsis patients. The paucity of effective treatments for sepsis-induced myopathy illustrates our incomplete understanding of the underlying disease pathophysiology. The expression patterns of sepsis-induced myopathy-associated genes must be identified to highlight potential regulatory targets.

Microarrays, which are routinely and broadly applied in clinical studies of human disease, allow researchers to profile changes in gene expression on a genome-wide scale. One caveat of microarray-based approaches is that genes selected for further analysis do not always play a key role in the process being studied. To overcome this obstacle, we designed and implemented a pattern-matching strategy based on Gene Set Enrichment Analysis (GSEA) [38, 40] to identify candidate genes. This approach generates a score for each gene to measure differences in expression between two gene sets by comparing their expression profiles. GSEA is an analytical method that uses gene sets representing different biological processes as a framework, within which gene expression data can be interpreted. This technique has been implemented in drug discovery studies using gene sets (also called gene signatures) affiliated with diseases to interpret expression data in the presence or absence of different drug treatments [41, 42]. This approach was also modified to assess the mode of actions of different drugs by comparing the expression data of host cells treated with different drugs [43].

In this study, we gathered publicly available gene expression data obtained from septic patients to evaluate differences in gene expression. One hundred and seven siRNA knockdowns, where one candidate gene of interest was specifically silenced, were also used in this analysis. The septic gene expression profiles were compared with the siRNA knockdowns to identify and validate new therapeutic targets for sepsis-induced myopathy. Genes in which siRNA silencing resulted in similar cellular tissue expression changes as in sepsis-induced myopathy patients were selected for further analysis.

Materials and methods

Computational assessment of distances between different expression data

We used a systematic computational approach based on the integration of publicly available sepsis-induced myopathy gene expression signatures with the gene expression signatures of individual siRNA-mediated gene knockdown experiments using candidate septic genes [44, 45]. The analytic workflow was shown in **Figure 1**. The expression profiles from sepsis-induced myopathy patients and siRNA perturbation were generated in the same platform (Affymetrix Human Genome U133 Plus 2.0 Array, GPL570). We systematically evaluated the gene expression signatures from sepsis-induced myopathy data sets derived from public microarray data against a collection of gene expression signatures from 107 different siRNA knockdown experiments to infer previously uncharacterized relationships between gene-disease pairs represented in the data sets. Probe rank lists of the same perturbation were combined into a single list according a hierarchical majority-voting scheme [46]. The differences between the sepsis-induced myopathy and siRNA knockdowns were calculated by comparing the corresponding ranked probe lists. We identified gene signatures for each probe list by selecting for the 250 probes with the highest and lowest values. We ranked the probes by considering the fold changes and values as previously described [42]. First, expression values of a pair of samples less than their primary threshold value (the lower quartile of expression values of these two samples) were set to the empirical value. Then, probes were ranked in descending order of corresponding experiment-to-control ratio values. For probes where the ratio value was equal to one, the secondary threshold value (one-tenth of primary threshold value) was used to reset the value of

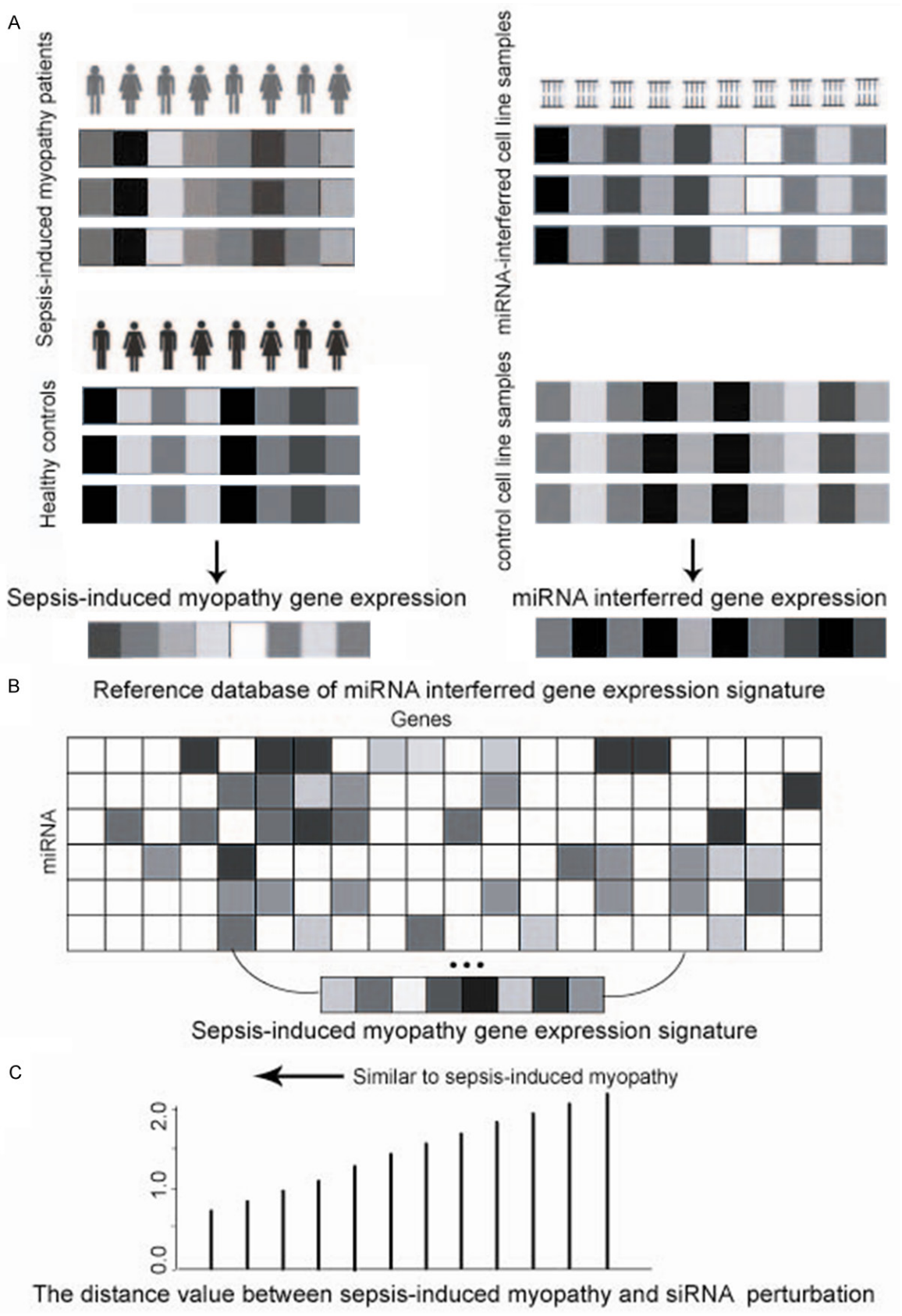

Figure 1. Analytic workflow. A. Two gene expression collections are used: a set of sepsis-induced myopathy associated gene data with corresponding controls and a set of gene expression data (One hundred and seven siRNA

knockdowns) from previously published experiments measuring cell lines knocked-down by siRNA with corresponding controls. Then, probes were ranked in descending order of corresponding experiment-to-control ratio values. B. Gene Set Enrichmet Analysis (GSEA) enrichment score of the signature of sepsis-induced myopathy were computed against the score in the ranked probe lists representing miRNA perturbation, and vice versa. C. The GSEA scores of sepsis-induced myopathy patients and siRNA perturbation cell lines were combined to generate a single value, which represented the distance between the two sets. A small distance value indicated that there were similar changes in gene expression. The calculated distance values between the sepsis and siRNA knockdown groups are displayed in **Table 1**.

Table 1. Calculated distance values between the sepsis-induced myopathy and siRNA knockdown groups. All siRNA knockdowns are listed by their target gene name

107siRNA-mediated knockdown	Distance value
PSIP1	0.846507
TOP1	0.864787
PCGF2	0.865381
SRF	0.875062
HIF1A	0.880559
FAS	0.887862
BCL11A	0.888228
UPF1	0.891073
PARK7	0.891151
MYST1	0.892264
SOX4	0.892325
CBFA2T3	0.892453
PTHLH	0.893483
TFAP2C	0.894412
SRA1	0.894728
TFAP2A	0.89512
SCYL1	0.899875
QKI	0.900248
TARDBP	0.903001
IGF2BP1	0.905588
YAP1	0.906771
HDAC5	0.908583
PROX1	0.909666
SMAD2	0.914027
EPAS1	0.91754
SMAD3	0.918948
NR2F2	0.919858
TP73L	0.927093
CIITA	0.92921
CCNT1	0.929703
ESR1	0.941071
NUDT6	0.941476
ETS1	0.944438
AHI1	0.945377
IFITM1	0.94726
CREB1	0.953022
WTAP	0.954409
BIRC4	0.954683
PROM1	0.957317
AOF2	0.959019
HOXC6	0.960384
ERG	0.962151
BMI1	0.962349
FOXF2	0.966985
MYC	0.968673
SUZ12	0.974166
STAU1	0.977303
RBM21	0.980872
NMNAT1	0.980922
EWSR1/FLI1	0.985703
NRIP1	0.989287
SNCA	0.990638
RHOBTB2	0.992023
EGR3	0.995776
VIL2	0.996416
KISS1R	0.997073
PIR	0.997578
POLRMT	0.999848
POU5F1	1.003346
WASF3	1.00788
FTMT	1.01178
SDHB	1.012574
MAF	1.014872
PIP5K1A	1.024446
E2F4	1.027148
RB1	1.027674
PAX3	1.029456
WT1	1.033928
CXCR4	1.035608
NRAS	1.040339
SOX2	1.043177
CDH11	1.046073
PDK2	1.0475
RBL2	1.050092
TP53	1.051123
NFE2L1	1.051851
ARHGDIB	1.052762
AR	1.054154

CKS1B	1.05835
DNMT1	1.058402
DSG2	1.062046
MCAM	1.064798
FSCN1	1.065549
EZH2	1.066153
AOF1	1.066587
FAP	1.07919
CDR2	1.07935
PTEN	1.079827
CCND1	1.080323
FOXM1	1.0813
BAHD1	1.082187
CDK4	1.082518
YY1	1.084885
CDX1	1.085265
YY2	1.088194
BRCA1	1.094276
PTK2	1.100451
DHX36	1.100753
BRAF	1.102154
NME2	1.103022
MMP14	1.112744
ALDH1A1/ALDH1B1	1.114988
MITF	1.11587
MYB	1.121352
PCSK9	1.121746
CTNNB1	1.13809
EED	1.159262

these probes. These probes were sub-sorted in descending order of the new ratio to produce the final probe rank list of each pair of sample. The septic myopathy and siRNA scores were then combined to generate a single value that represented the distance between septic myopathy and siRNA-mediated knockdown.

Animals and experimental protocol

Pathogen-free, male C57BL/6 mice were used for all mouse experiments. The mice ranged in age from nine to 10 weeks and were between 22 and 25 g. All mice were raised on standard rodent chow and water ad libitum for at least one week prior to the start of experiments. Polymicrobial peritonitis was induced using the cecal ligation and puncture (CLP) approach [47]. Briefly, a 1 cm abdominal midline incision was made to expose the cecum, which was then ligated and punctured using a 23-gauge needle. To ensure that the puncture was successful, a small amount of feces was extruded from the cecum. As a sham control, mice were subjected to an abdominal incision but cecal ligation and puncture were not performed. Following the puncture, the cecum was returned to the abdominal cavity, at which point the abdominal muscles and skin were closed. Five days after CLP, mice were anesthetized with a combination of ketamine and xylazine, the gastrocnemius muscles were excised and the mice were euthanized. Each experiment was performed in triplicate. An endpoint at five days was chosen as preliminary studies showed that this was the earliest point at which a net decrease in muscle weight was detectable in septic mice.

Table 2. Primers used for quantitative RT-PCR assays

Gene names	Sequences
mo-PSIP1(+)	GCCTCTTTGTAATAATGCGTAG
mo-PSIP1(-)	ATAAAGGATTGTAGGTGGACT
mo-TOP1(+)	GGTTACTTGGCTGGTCTCCT
mo-TOP1(-)	CGATACTGATTTCGGATCTTGT
mo-PCGF2(+)	GACACTTCCCAAATCTCCTC
mo-PCGF2(-)	CAATGGTGGTGGCATCAATA
mo-SRF(+)	GGGACGGCACCACTTATT
mo-SRF(-)	GCAGAGCAGGAAGGAGGG
mo-HIF1A(+)	GGTATTATTCAGCACGAC
mo-HIF1A(-)	CAGCTTGTATCCTCTGATT
mo-FAS(+)	GCAAACCAGACTTCTACTGCGA
mo-FAS(-)	TTTGTATTGCTGGTTGCTGTGC
mo-UPF1(+)	GCGCACAGCTGAGAGAGAACT
mo-UPF1(-)	TCACCAGCACCCACACATGT
mo-PARK7(+)	CATACGATGTGGTGGTTCTT
mo-PARK7(-)	CCATAGGCGACTCAGATAAA

The Institutional Animal Care and Use Committee of Jilin University approved all studies involving mice in this study.

RNA extraction and real-time RT-PCR quantification

Total RNA was isolated from the mouse gastrocnemius using the TRIzol reagent (Life technologies, Carlsbad, CA, USA) according to the manufacturer's specifications. cDNA was synthesized using 2.5 μg of total RNA in a 50 μl reaction using reverse transcriptase (TaKaRa, Dalian, China). Primers (**Table 2**) for the following targets were synthesized by Invitrogen (Shanghai, China): PSIP1, TOP1, PCGF2, SRF, HIF1A, FAS, UPF1 and PARK7. Real-time RT-PCR

quantification for individual mRNAs was performed on an ABI Model 7500 Sequence Detector (Applied Biosystems Inc., Foster City, CA, USA) using the TaKaRa real-time PCR kit. All RT-PCR reactions were performed using the following parameters: 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 34 s and 72°C for 15 s. The cycle threshold (CT) for each target product and HPRT were quantified. The $2^{-\Delta\Delta CT}$ was calculated and used for quantification and statistical analyses (the results were expressed as fold change relative to controls).

Statistical analysis

All results are expressed as the mean ± standard deviation from three separate experiments. A one-way ANOVA was used to measure differences between the groups. All statistical analyses were performed using SPSS, version 16.0 (SPSS, Chicago, IL, USA). A *P*-value of <0.05 was set as the threshold for statistical significance.

Results

Prediction of septic myopathy-related genes

We identified and compared the publicly available gene expression data from septic myopathy patients with siRNA knockdown experiments listed in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) [44, 45]. To fully exploit the collected expression data, we only used data that was obtained on the Affymetrix Human Genome U133 Plus 2.0 Array (GPL570) platform. The GSE5081 series of septic myopathy samples were ultimately selected for this study. Additionally, 27 studies containing 107 different siRNA knockdowns were selected for the siRNA group. The relationship between the expression changes in each group was evaluated as a distance between zero and two. We calculated the distances between the septic myopathy group and the siRNA knockdown group (**Table 1**).

PSIP1 gene expression is decreased in mice with septic myopathy

To validate candidate genes identified from the comparison of the human septic myopathy and siRNA knockdown groups, we selected the top eight genes (PSIP1, TOP1, PCGF2, SRF, HIF1A, FAS, UPF1 and PARK7) whose siRNA knockdown had the smallest distance from the septic myopathy group and examined their expression levels in the CLP mouse model of septic myopathy. As shown in **Figure 2**, the expression of PSIP1 decreased (56% decrease) in mice with septic myopathy, while HIF1A, FAS and UPF1 expression increased in septic myopathy mice. There was no difference in expression of TOP1, PCGF2, SRF and PARK7 between the control and the septic mopathy groups.

Discussion

Using an *in silico* approach based on the integration of publicly available gene expression data, we analyzed the differences between septic patient gene expression data sets and siRNA knockdown to determine which genes may serve as therapeutic targets for treatment. We selected eight genes, which were our top candidates, and found that PSIP1 was significantly decreased in both the array comparison and our CLP assay.

We hypothesize that PSIP1 is a potential new target gene for sepsis-induced myopathy, and performed experimental validation to confirm that PSIP1 was decreased in a CLP mouse model of sepsis. PSIP1 (also known lens epithelium derived growth factor (LEDGF/p52) is a member of the hepatoma-derived growth factor (HDGF) family, but its function is currently unknown. Although PSIP1 has not previously been associated with sepsis-induced myopathy, it has been shown to repress the transcriptional activity of MeCP2 [48, 49]. MeCP2 is a cellular interacting partner of both LEDGF/p75 (also known as PSIP2) and LEDGF/p52, and a transactivator of Hsp27pr. Bueno et al. [50] recently reported that mutations impairing SUMOylation of LEDGF/p75 increase its transcriptional activity, but not that of PSIP1, suggesting that these splice variants activate Hsp27pr via different molecular mechanisms. The splice variants have antagonistic functions, with LEDGF/p75 having a pro-survival effect and LEDGF/p52 playing a pro-apoptotic role [48]. Because a mouse knockout for PSIP1 has not yet been generated, additional studies are warranted to determine if PSIP1, PSIP2, and MeCP2 are part of a larger dynamic transcriptional complex that regulates the expression gene networks in muscles during sepsis.

Future studies should determine the precise mechanism of action by which PSIP1 contrib-

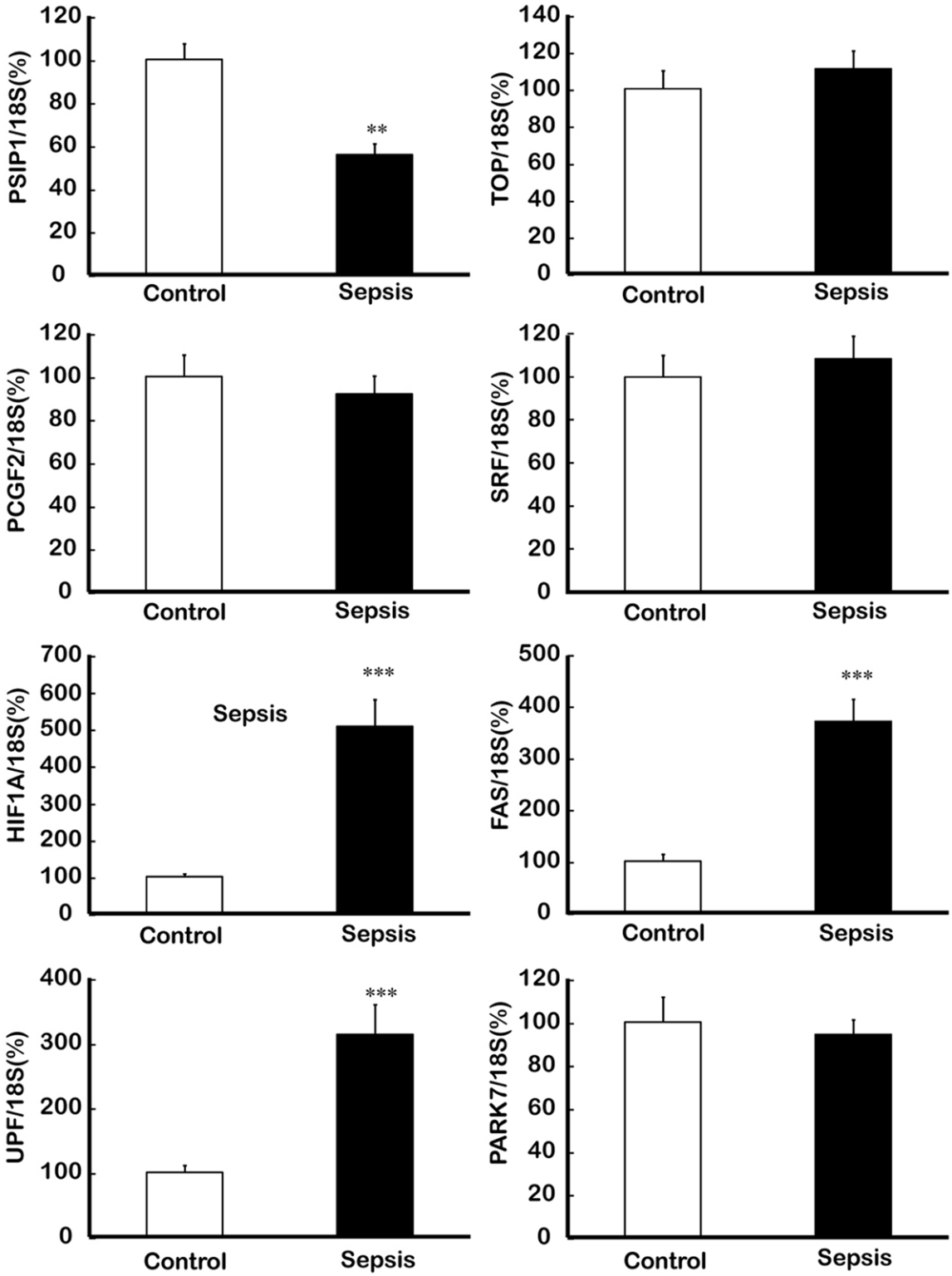

Figure 2. PSIP1, TOP1, PCGF2, SRF, HIF1A, FAS, UPF1 and PAPK7 mRNA content in skeletal muscle obtained from nonseptic (control) and CLP-induced septic mice. Values were normalized to the 18S ribosomal subunit. The nonseptic control values were set at 100% arbitrary units (AUs). Values are Presented as the mean ± SEM; n=6 animals/group. The asterisk indicates a significant difference ($P<0.01$) when compared to the control group. Expression of PSIP1 was decreased in the septic group, whereas HIF1A, FAS and UPF1 expression were increased. The expression levels of TOP1, PCGF2, SRF and PARK7 were not changed between the control and experimental groups.

utes to the pathophysiology of sepsis-induced myopathy through western blotting to examine changes in PSIP1 protein expression and immunohistochemistry to examine changes in protein localization during sepsis. Further investigation into this target gene could yield novel insights into the diagnosis, treatment or prevention of sepsis-induced myopathy and other critical illness myopathies.

Although the experimental results for PSIP1 in our rodent model of sepsis-induced myopthy support our computational predictions derived from gene expression measurements in humans, there are several caveats that limit the interpretation of the results. While we initially selected candidate genes that were decreased in human septic myopathy patients and the siRNA knockdowns, TOP1, PCGF2, SRF, and PARK7 expression levels were unchanged while HIF1A, FAS and UPF1 expression levels were elevated after CLP sepsis induction. Because we only tested our predicted target genes in one model of septic myopathy, it may not fully recapitulate sepsis-induced myopathy in humans. Although CLP is the most widely used model for human sepsis-induced myopathy, and previous molecular studies have established that it shares many molecular characteristics of human septic myopathy, this model is not suitable for long-term studies, indicating that a model of severe chronic sepsis characterized by persistent hypermetabolism is still greatly needed [51]. In this experiment, we only tested some gene expression levels on the gastrocnemius muscles of mice sepsis model. We are not sure that the target gene express level in muscle or other tissues are consist with in blood cells. The blood sampls are very easy to get from patients with sepsis-induced myopathy. It will be difficult for us to get muscle tissue samples from patients with sepsis-induced myopathy.

Only four of the eight genes from the human septic myopathy gene signature selected for PCR validation in the rodent model showed a statistically significant difference between the control and septic myopathy groups. This may be due to species-specific differences in gene expression between human muscle tissue used to select the genes and the rat muscle tissue used for validation. Moreover, several of these genes may reach significantly different expression levels only after a longer period of disease. Therefore, additional studies with alternative models of septic myopathy are needed to evaluate the other predicted target genes.

Here, we have shown that computational approaches leveraging public gene expression microarray data can be used to identify potential target genes for sepsis-induced myopathy. We show a decrease in PSIP1 expression in mice with septic myopathy, which indicates that it is possible to identify target genes involved in the pathophysiology and treatment of sepsis-induced myopathy. These findings support the need for future studies that use computational approaches to leverage publicly available molecular data to identify target therapeutics for additional diseases.

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Disclosure of conflict of interest

None.

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