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Intensive Care Unit Acquired Weakness genes exist between non-survivors and survivors in patients with sepsis

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B.S., Tokyo Metropolitan University, 2010

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An abstract of
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Abstract

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Sepsis is a potentially lethal condition, and intensive care unit (ICU) acquired weakness (ICUAW) is one of the complications of sepsis. Bioenergy failure is a plausible mechanism of organ failure and ICUAW, but the link between sepsis-related mortality and ICUAW remains unclear. Elucidating this biological link is crucial to the understanding of the pathophysiology of sepsis-related mortality. Using pre-identified ICUAW genes, a publicly available gene expression profile GSE54514 containing whole blood samples within 24 hours of sepsis onset was analyzed. Differential gene expression analysis identified 38 genes significantly different between 8 non-survivors and 13 survivors with primary bacteremia- and respiratory-triggered sepsis. Functional enrichment analysis of differentially expressed ICUAW genes identified impaired cadherin binding, sarcomere formation, and energy metabolism among non-survivors. Further, we interrogated ICUAW genes in patients with respiratory-triggered sepsis. In this population, a deficit in energy metabolism, cadherin binding, sarcomere formation, and granule secretion was observed. Our findings demonstrated an association between ICUAW genes and sepsis-related mortality in the early phase of sepsis. Finally, defects in energy metabolism and muscle fiber formation are likely resulting in septic patients who are non-survivors, especially in respiratory-triggered sepsis.

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Introduction

Sepsis is a life-threatening syndrome and a major public health concern [1]. Globally, 19.7% of death in the world is attributed to sepsis [2]. During sepsis, an infectious agent triggers Pathogen-Associated Molecular Patterns (PAMPs) through Pattern Recognition Receptors (PRR), leading to systemic inflammation. This pathway is further exacerbated by Damage-Associated Molecular Patterns (DAMPs) [3]. This vicious cycle inflicts Persistent inflammation, Immunosuppression, and catabolism syndrome (PICS) [3]. While the recent sepsis-3 criteria define sepsis as an organ dysfunction due to a dysregulated host response, organ failure is not characterized by a histological change [4]. Mounting evidence shows mitochondrial dysfunction acts as a key role in organ failure [5–9]. Mitochondria produce energy for a cell by producing adenosine triphosphate (ATP) by oxidative phosphorylation. During sepsis, inflammation induces mitochondrial dysfunction caused by insufficient hemodynamic perfusion and mitochondrial malformation [6]. In addition, sustained inflammatory signaling suppresses metabolic enzymes [5, 10]. Both lead to organ failure with few signs of cell death [4, 6].

Intensive Care Unit Acquired Weakness (ICUAW) is a syndrome of limb muscle weakness, and one of the sequelae of sepsis [11, 12]. ICUAW gene expression signature was discovered from muscle biopsies of adult septic patients with ICUAW, and the main culprit behind ICUAW is a global energy failure, which is characterized by deranged mitochondrial respiration [13]. Mitochondrial capability in a muscle in septic patients has an inverse association with sepsis severity [14]. In addition, clinical epidemiology demonstrates that ICUAW is associated with a higher risk of in-hospital mortality [15–17]. This raises a question as to whether biological mechanisms of sepsis-related mortality and ICUAW are overlapped. Specifically, ICUAW genes associated with sepsis-related mortality have not been explored.

In this work, the primary objective was to investigate whether the pre-selected ICUAW genes were present between non-survivors and survivors within 24 hours of sepsis onset. Our secondary aim was to explore novel mechanistic pathways relating to mortality. Because mortality varies depending on

the site of infection [18], we anticipated ICUAW-associated genes may be biased by site of infection. Finally, we further assessed ICUAW-associated genes relating to mortality based on the reported site of infection.

Materials and methods

Study design and population

This is a secondary analysis of publicly available Gene Expression Omnibus (GEO) microarray data, GSE54514, consisting of whole blood samples from septic adults compared with healthy controls [19]. In this dataset, sepsis was defined by sepsis-2 criteria [20]. This dataset consists of patients with primary bacteremia-, respiratory-, urinary tract-triggered sepsis, and unavailable site of infection described as "others" in the original study.

We excluded patients with urinary tract-triggered sepsis and the "others" class because of unbalanced or nonexistent cases related to mortality, remaining primary bacteremia- and respiratory-triggered sepsis in the primary analysis. The original study characteristics are shown in the **Supplementary table 1**. In the dataset, longitudinal data were available from the first day to 5 days after sepsis onset. To avoid loss to follow up due to death and observe gene expression and its pathway in the early phase of sepsis, we used day 1 data in this analysis.

Microarray data

The GSE54514 dataset was processed by normalization and log-transformation, and no batch effect was confirmed. For quality control, we excluded genes expressed in fewer than three samples, genes with an expression less than the median level gene expression for all genes in the dataset, and genes without an Entrez Gene ID. For Entrez Gene ID with multiple probes, we calculated the median expression value of all corresponding probes to determine the expression value for each gene. A total of 13,972 genes were available in this analysis.

ICUAW-associated genes

Walsh et al. [13] identified 695 genes from 14 sepsis patients with mechanical ventilation for at least a week and who were diagnosed as ICUAW post ICU discharge and 8 healthy controls. We verified that this is the only available dataset to allow for testing our hypothesis. Among 695 ICUAW-associated genes, we excluded genes that were unavailable or duplicated by their Entrez Gene ID, with 526 remaining for analysis.

Differential gene expression analysis

Age-adjusted differential gene expression analysis among ICUAW-associated genes was performed in the sepsis microarray dataset between non-survivors and survivors, using the R limma package with a Benjamini-Hochberg (BH) correction method and adjusted p value <0.05 [21]. In addition, a biological significant threshold was set to an absolute value of log2(fold change) >0.5. Significantly overrepresented genes were clustered by hierarchical clustering in R pheatmap package (version 1.0.12).

Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to identify overrepresented GO categories and KEGG pathways for differentially expressed ICUAW-associated genes in septic patients between non-survivors and survivors. We used the R clusterProfiler package with BH correction for GO analysis to identify Biological Process (BP), Cellular Component (CC), and Molecular Function (MF), and to perform KEGG pathway enrichment analysis of the differentially expressed genes using adjusted p value <0.05 [22].

We anticipated a confounding effect on the association between ICUAW-associated genes and sepsis-related mortality due to the site of infection. Thus, we performed stratified analysis based on the site of infection. However, due to the small sample size in patients with primary bacteremia-triggered sepsis, we only analyzed patients with respiratory-triggered sepsis in the secondary analysis using differential gene expression and functional enrichment analysis.

Due to the small sample size and unbalanced characteristics in the original dataset, we did not include urinary tract-triggered sepsis in the primary analysis and primary bacteremia triggered-sepsis in the secondary analysis. The characteristics and results of differential gene expression analysis and functional enrichment analysis are shown in **Supplementary Table 1, Figure 1, and Figure 2**. Data acquisition and statistical analyses were performed using R (version: 4.0.1).

Results

Subject characteristics

Of 21 septic patients with the microarray data, 13 (61.9%) were survivors and 8 (38.1%) were non-survivors. The median and the interquartile range (IQR) of age among survivors was 60.0 years (IQR:52.0-68.0) and 69.0 years (IQR:61.5-80.0) among non-survivors. Demographics and clinical characteristics of the study population are shown in **Table 1**.

Differential gene expression analysis

Of the 526 genes available for analysis, a total of 38 genes were differentially expressed between non-survivors and survivors within 24 hours after sepsis onset. As shown in the volcano plot in **Figure 1-a**, a total of 15 genes were up-regulated and 23 genes were down-regulated among non-survivors. These 38 differentially expressed ICUAW-associated genes were clustered into non-survivors and survivors in the heatmap shown in **Figure 1-b**.

Functional enrichment with GO and KEGG pathway enrichment analysis

Functional enrichment analysis using the 38 differentially expressed genes is shown in **Figure 1-c**. Significantly up-regulated genes enriched membrane component pathways. Down-regulated genes enriched cadherin binding, sarcomere formation, and energy metabolism including nicotinamide adenine dinucleotide reduced form (NADH) and glycolysis. KEGG pathway enrichment analysis identified 3 pathways involved in the biosynthesis of amino acids, hypoxia-inducible factor (HIF)-1 signaling, and carbon metabolism with down-regulated genes (**Figure 1-d**).

Restriction to respiratory-triggered sepsis

Of 526 available ICUAW-associated genes, 50 genes were significantly expressed in respiratory-triggered sepsis between non-survivors and survivors. The volcano plot and heatmap are shown in **Figure 2-a, b**.

Using functional enrichment analysis, up-regulated genes in patients with respiratory-triggered sepsis enriched membrane components, amino acid, and ion transportation. Down-regulated genes enriched NADH, glycolysis, cadherin binding, granule secretion, and sarcomere formation (A and M band) (**Figure 2-c**). All GO terms identified in respiratory-triggered sepsis are listed in **Supplementary Table 2**. KEGG pathway enrichment analysis identified 4 pathways involved in the biosynthesis of amino acids, HIF-1 signaling, carbon metabolism, and glycolysis/gluconeogenesis with down-regulated genes (**Figure 2-d**).

Discussion

We used pre-selected ICUAW-associated genes from an earlier experiment to observe a pathophysiological link between sepsis-related mortality and ICUAW from the blood within 24 hours of sepsis onset. We observed 38 ICUAW-associated genes were differentially expressed between non-survivors and survivors. Functional enrichment analysis of these differentially expressed ICUAW-associated genes identified a failure in sarcomere formation and bioenergy production.

In sepsis, an energy metabolism switches from oxidative phosphorylation to glycolytic metabolism, known as the Warburg effect [23]. Energy failure in oxidative phosphorylation is associated with sepsis-related mortality [14]. For ICUAW, although acute differential gene expression within 24 hours after sepsis onset has not been studied, expression profiling of mitochondrial components has shown that changes in bioenergy failure from day 1 to 7 in critically ill patients was associated with muscle mass reduction [24]. When compared with healthy controls, mitochondria and bioenergy metabolism were down-regulated in patients with sepsis who were diagnosed with ICUAW after ICU discharge [13]. This bioenergy failure is explained by prolonged inflammation, known as Persistent

Inflammation, Immunosuppression, and Catabolic Syndrome [25]. In our study, down-regulated ICUAW-associated genes enriched the NADH regeneration. This suggests an energy failure, specifically impaired mitochondrial respiration in ICUAW, may be present in septic patients who are non-survivors.

Although glycolytic energy generation is a counterpart to aerobic respiration, the rate of glycolysis did not significantly differ between chronic critical ill patients with ICUAW and healthy controls [26]. While few studies reported disrupted glycolysis in ICUAW, broad defects in energy metabolism, characterized by impaired oxidative phosphorylation and glycolysis, underlie sepsis-induced immunometabolic paralysis [27]. In the immunometabolic paralysis phase of sepsis, both HIF-1 signaling, and glycolytic metabolism are down-regulated and energy metabolism shifts toward fatty acid oxidation [28]. Our analysis showed a down-regulation of glycolysis in septic non-survivors, suggesting those non-survivors may have both impaired oxidative phosphorylation and glycolysis in blood within 24 hours of sepsis onset.

Mortality differs based on the site of infection [18], but the difference in pathophysiology based on site of infection has not been explored. Due to the limited characteristics of the available data, we analyzed respiratory-triggered sepsis. In this analysis, disrupted global energy metabolism and the HIF-1 signaling pathway were identified. This suggests immunometablic paralysis occurred in respiratory sepsis patients who were non-survivors. In addition, genes related to sarcomere formation were impaired. It suggests that muscle fiber malformation may occur among non-survivors in patients with respiratory-triggered sepsis, and they may be susceptible to muscle weakness.

Our study has several limitations. First, due to the limited sample size and available population characteristics, unmeasured confounders may produce biased genes between non-survivors and survivors. In our stratified analysis, the limited sample size in primary bacteremia- or urinary tract-triggered sepsis prevents analyzing these populations to interrogate ICUAW-associated genes across site of infection. Second, ICUAW-associated genes were identified by muscle biopsy, which is impractical and not performed as part of routine clinical care. Therefore, our analysis was conducted

using whole blood samples; however, we acknowledge that tissue-specificity may show biased estimates of significant genes.

In conclusion, we identified an association between ICUAW-associated genes and sepsis-related mortality from the blood within 24 hours of the sepsis onset. Exploration of these ICUAW-associated genes identified derangement in metabolic energy-producing pathways and sarcomere formation. These pathways appeared in patients with respiratory-triggered sepsis. Further investigation of the biological link between sepsis-related mortality and ICUAW across site of infection is warranted.

Data availability

R scripts used for data preprocessing, and analysis are available from https://github.com/seibikobara/ICUAW_DEGs. Datasets generated and analyzed during the study are available upon request.

Reference

1 Singer, M. *et al.* The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA **315,** 801-810 (2016).

2 Rudd, K. E. *et al.* Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. The Lancet **395,** 200-211 (2020).

3 van der Poll, T., van de Veerdonk, F. L., Scicluna, B. P. & Netea, M. G. The immunopathology of sepsis and potential therapeutic targets. Nat Rev Immunol **17,** 407-420 (2017).

4 Hotchkiss, R. S. & Karl, I. E. The pathophysiology and treatment of sepsis. N Engl J Med **348,** 138-150 (2003).

5 Lee, I. & Huttemann, M. Energy crisis: the role of oxidative phosphorylation in acute inflammation and sepsis. Biochim Biophys Acta **1842,** 1579-1586 (2014).

6 Singer, M. The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. Virulence **5,** 66-72 (2014).

7 Arulkumaran, N. *et al.* Mitochondrial Function in Sepsis. Shock **45,** 271-281 (2016).

8 Maestraggi, Q. *et al.* Skeletal Muscle and Lymphocyte Mitochondrial Dysfunctions in Septic Shock Trigger ICU-Acquired Weakness and Sepsis-Induced Immunoparalysis. Biomed Res Int **2017,** 7897325, doi:10.1155/2017/7897325 (2017).

9 Rahmel, T. *et al.* Mitochondrial dysfunction in sepsis is associated with diminished intramitochondrial TFAM despite its increased cellular expression. Sci Rep **10,** 21029, doi:10.1038/s41598-020-78195-4 (2020).

10 Deutschman, C. S. & Tracey, K. J. Sepsis: current dogma and new perspectives. Immunity **40,** 463-475 (2014).

11 Stevens, R. D. *et al.* A framework for diagnosing and classifying intensive care unit-acquired weakness. Crit Care Med **37,** S299-308 (2009).

12 Fan, E. et al. An official American Thoracic Society Clinical Practice guideline: the diagnosis of intensive care unit-acquired weakness in adults. Am J Respir Crit Care Med **190,** 1437-1446 (2014).

13 Walsh, C. J. *et al.* Transcriptomic analysis reveals abnormal muscle repair and remodeling in survivors of critical illness with sustained weakness. Sci Rep **6,** 29334, doi:10.1038/srep29334 (2016).

14 Brealey, D. *et al.* Association between mitochondrial dysfunction and severity and outcome of septic shock. The Lancet **360,** 219-223 (2002).

15 Garnacho-Montero, J. *et al.* Critical illness polyneuropathy: risk factors and clinical consequences. A cohort study in septic patients. Intensive Care Med **27,** 1288-1296 (2001).

16 Sharshar, T. *et al.* Presence and severity of intensive care unit-acquired paresis at time of awakening are associated with increased intensive care unit and hospital mortality. Crit Care Med **37,** 3047-3053 (2009).

17 Wieske, L. *et al.* Impact of ICU-acquired weakness on post-ICU physical functioning: a follow-up study. Crit Care **19,** 196, doi:10.1186/s13054-015-0937-2 (2015).

18 Chou, E. H. *et al.* Incidence, trends, and outcomes of infection sites among hospitalizations of sepsis: A nationwide study. PLoS One **15,** e0227752, doi:10.1371/journal.pone.0227752 (2020).

19 Parnell, G. P. *et al.* Identifying key regulatory genes in the whole blood of septic patients to monitor underlying immune dysfunctions. Shock **40,** 166-174 (2013).

20 Levy, M. M. *et al.* 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. Crit Care Med **31,** 1250-1256 (2003).

21 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res **43,** e47, doi:10.1093/nar/gkv007 (2015).

22 Yu, G., Wang, L. G., Han, Y. & He, Q. Y. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS **16,** 284-287 (2012).

23 Venet, F. & Monneret, G. Advances in the understanding and treatment of sepsis-induced immunosuppression. Nat Rev Nephrol **14,** 121-137 (2018).

24 Puthucheary, Z. A. *et al.* Metabolic phenotype of skeletal muscle in early critical illness. Thorax **73,** 926-935 (2018).

25 Hawkins, R. B. *et al.* Chronic Critical Illness and the Persistent Inflammation, Immunosuppression, and Catabolism Syndrome. Front Immunol **9,** 1511, doi:10.3389/fimmu.2018.01511 (2018).

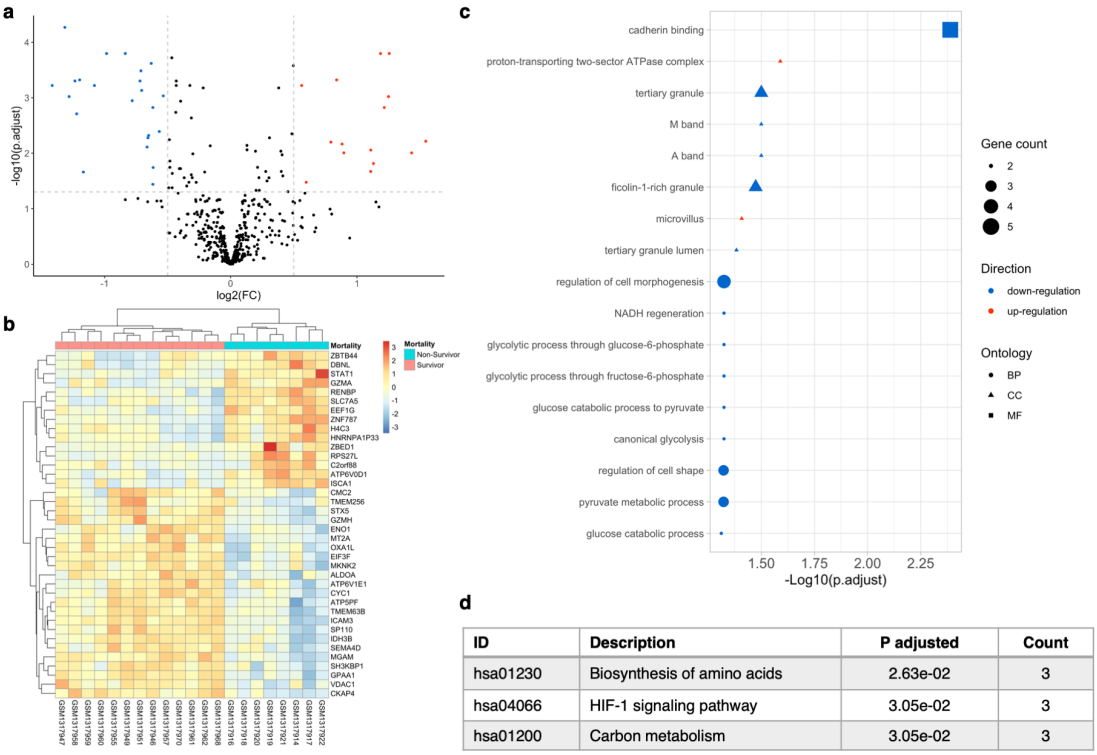
26 Jiroutkova, K. *et al.* Mitochondrial Function in an In Vitro Model of Skeletal Muscle of Patients With Protracted Critical Illness and Intensive Care Unit-Acquired Weakness. JPEN J Parenter Enteral Nutr **41,** 1213-1221 (2017).

27 Cheng, S. C. *et al.* Broad defects in the energy metabolism of leukocytes underlie immunoparalysis in sepsis. Nat Immunol **17,** 406-413 (2016).

28 Fitzpatrick, S. F. Immunometabolism and Sepsis: A Role for HIF? Front Mol Biosci **6,** 85, doi:10.3389/fmolb.2019.00085 (2019).

Table 1. Characteristics and demographics of the study population. APACHE II: Acute Physiology and Chronic Health Evaluation II.

Characteristics	**Survivors (n=13)**	**Non-survivors (n=8)**
Age, years (IQR)	60.0 (52.0-68.0)	69.0 (61.5-80.0)
APACHE II (IQR)	17.0 (15.0-24.0)	23.5 (19.75-26.5)
Female, n (%)	9 (69.2)	4 (50.0)



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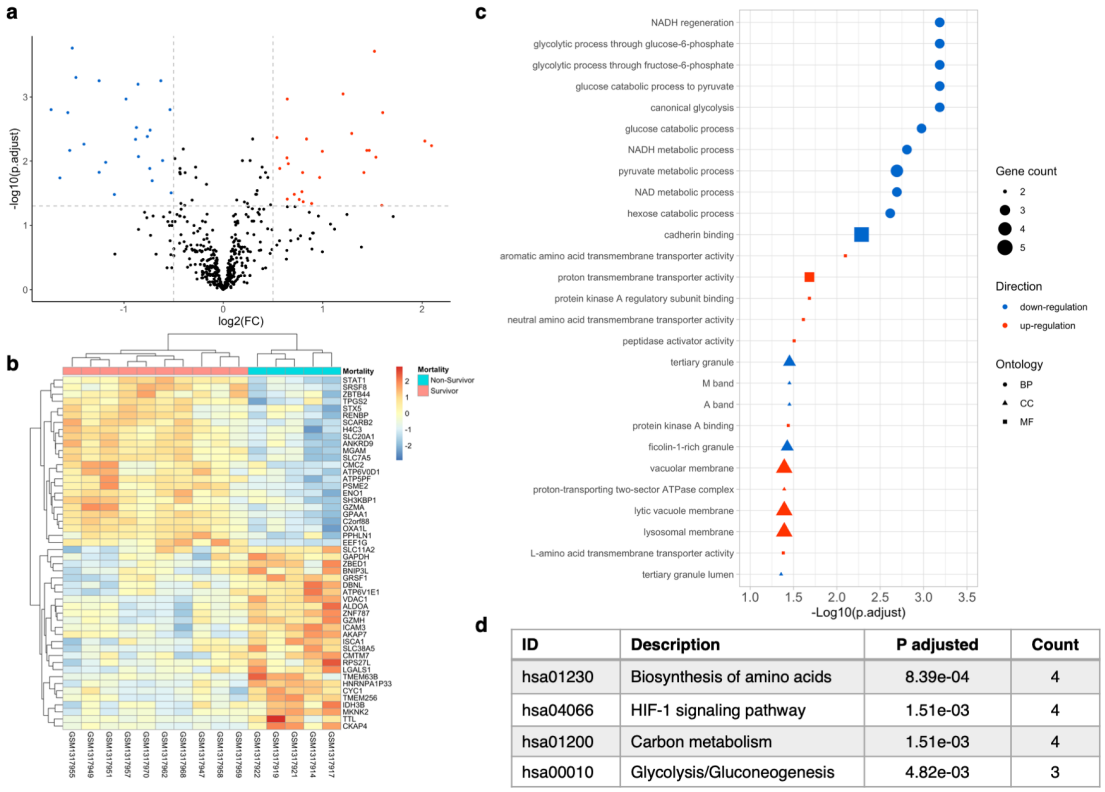
ID	Description	P adjusted	Count
hsa01230	Biosynthesis of amino acids	2.63e-02	3
hsa04066	HIF-1 signaling pathway	3.05e-02	3
hsa01200	Carbon metabolism	3.05e-02	3

Fig.1. Differential gene expression analysis of patients with sepsis. **(a)** Volcano plot of differentially expressed ICUAW-associated genes (ICUAW DEGs) between non-survivors and survivors in patients with sepsis. ICUAW DEGs were assessed at an adjusted p value <0.05 and absolute value of log2(fold change) >0.5. Of the 38 ICUAW DEGs, 15 genes were up-regulated (red dots) and 23 genes were down-regulated (blue dots). **(b)** Heat map of 38 ICUAW DEGs with mortality as an annotation. **(c)** Gene Ontology (GO) analysis includes the biological process (BP), cell component (CC), and molecular function (MF). Up-regulated ICUAW DEGs enriched membrane component pathways. Down-regulated ICUAW DEGs enriched cadherin binding, sarcomere formation, and energy metabolism. **(d)** Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis identified the biosynthesis of amino acids, hypoxia-inducible factor (HIF)-1 signaling, and carbon metabolism with down-regulated ICUAW DEGs.



d

ID	Description	P adjusted	Count
hsa01230	Biosynthesis of amino acids	8.39e-04	4
hsa04066	HIF-1 signaling pathway	1.51e-03	4
hsa01200	Carbon metabolism	1.51e-03	4
hsa00010	Glycolysis/Gluconeogenesis	4.82e-03	3

Fig.2. Differential gene expression analysis in patients with respiratory-triggered sepsis. (a) Volcano plot of differentially expressed ICUAW-associated genes (ICUAW DEGs) between non-survivors and survivors in patients with respiratory-triggered sepsis. Of the 50 ICUAW DEGs, 26 genes were up-regulated (red dots) and 24 genes were down-regulated (blue dots). **(b)** Heat map of 50 ICUAW DEGs with mortality as an annotation. **(c)** Gene Ontology (GO) analysis includes the biological process (BP), cell component (CC), and molecular function (MF). Up-regulated ICUAW DEGs in patients with respiratory-triggered sepsis enriched membrane component, amino acid, and ion transportation. Down-regulated ICUAW DEGs enriched NADH, glycolysis, cadherin binding, granule secretion, and sarcomere formation (A and M band). **(d)** Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis identified the biosynthesis of amino acids, HIF-1 signaling, carbon metabolism, and glycolysis/gluconeogenesis with down-regulated ICUAW DEGs.