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mRNA–miRNA integration analysis of T-cell exhaustion in sepsis from community-acquired pneumonia

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Abstract

Aim: Community-acquired pneumonia is an acute lung infection in patients without recent healthcare exposure that can progress to severe sepsis. Despite the well-established influence of miRNAs on inflammation, their specific roles in pneumonia-associated sepsis remain underexplored. In this pilot study, we aimed to provide insights into the pathogenesis of community-acquired pneumonia-associated sepsis by performing an integrative mRNA–miRNA analysis to identify key cellular signaling pathways and potential molecular targets for future research and treatment development.

Methods: We conducted a prospective, observational, single-center study including 14 critically ill patients with community-acquired pneumonia-associated sepsis and 15 healthy controls (median age: 78 [interquartile range 67.3–83.5] and 55 [interquartile range 40.5–59.0] years, respectively).

Results: Eleven patients required ventilatory support, and six met the diagnostic criteria for septic shock. All patients survived. RNA sequencing revealed 1209 upregulated and 1461 downregulated differentially expressed genes for mRNAs (false discovery rate < 0.05, $|\log_2$ fold change$|$ >1.2), 51 upregulated and 21 downregulated genes for miRNAs, and 646 upregulated and 1274 downregulated for mRNA related to miRNAs. Canonical pathway analysis revealed activation of the programmed death-1/programmed death-ligand-1 cancer immunotherapy pathway and suppression of the Th1 pathway, indicating T-cell exhaustion in the acute phase of community-acquired pneumonia-associated sepsis.

Conclusion: This study provides valuable insights into the molecular mechanisms underlying CAP-associated sepsis, confirming the occurrence of immune dysregulation, particularly T-cell exhaustion. Our findings suggest that specific miRNAs and signaling pathways identified here may serve as potential therapeutic targets or biomarkers.

KEYWORDS
community-acquired pneumonia, microRNAs, mRNAs, PD-1/PDL1, T-cell exhaustion

INTRODUCTION

Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, is most often attributed to pneumonia, accounting for up to half of all sepsis cases.[1] Pneumonia, a common acute respiratory infection, affects the alveoli and distal bronchial tree and remains a leading cause of morbidity and mortality worldwide.[1] Community-acquired pneumonia (CAP) is an acute lung infection involving the alveoli in individuals without recent healthcare exposure.[2] Therefore, to investigate the pathogenesis of sepsis, we focused on patients with CAP-associated sepsis.

The transcribed mRNAs translate proteins, which lead to a progressive inflammatory response that can lead to fatal disseminated intravascular coagulation syndrome and multiorgan damage.[3] MicroRNAs (miRNAs) are short, single-stranded RNA fragments comprising 21–23 bases that play crucial roles in maintaining homeostasis and regulating cellular physiological functions. MiRNAs are a class of endogenous small regulatory RNA molecules that target mRNAs and trigger either translation repression or mRNA degradation,[4] which is called mRNA interference. However, not all mRNAs serve as direct targets of miRNAs. Therefore, in this study, we specifically focused on mRNAs, miRNAs, and mRNA-related miRNAs to investigate the pathogenesis of the condition through the lens of mRNA interference. Recently, mRNA–miRNA integration analyses have become instrumental in exploring the pathological mechanisms of various chronic inflammatory diseases such as breast cancer.[4] This analysis has also been used for acute inflammatory diseases such as inflammatory bowel disease.[5] However, to our knowledge, the application of mRNA–miRNA integration analysis to study CAP-associated sepsis remains underexplored. Therefore, in this study, we directly measured mRNAs, miRNAs, and miRNA-related mRNAs to evaluate the pathogenesis of the condition through the lens of mRNA interference. Through

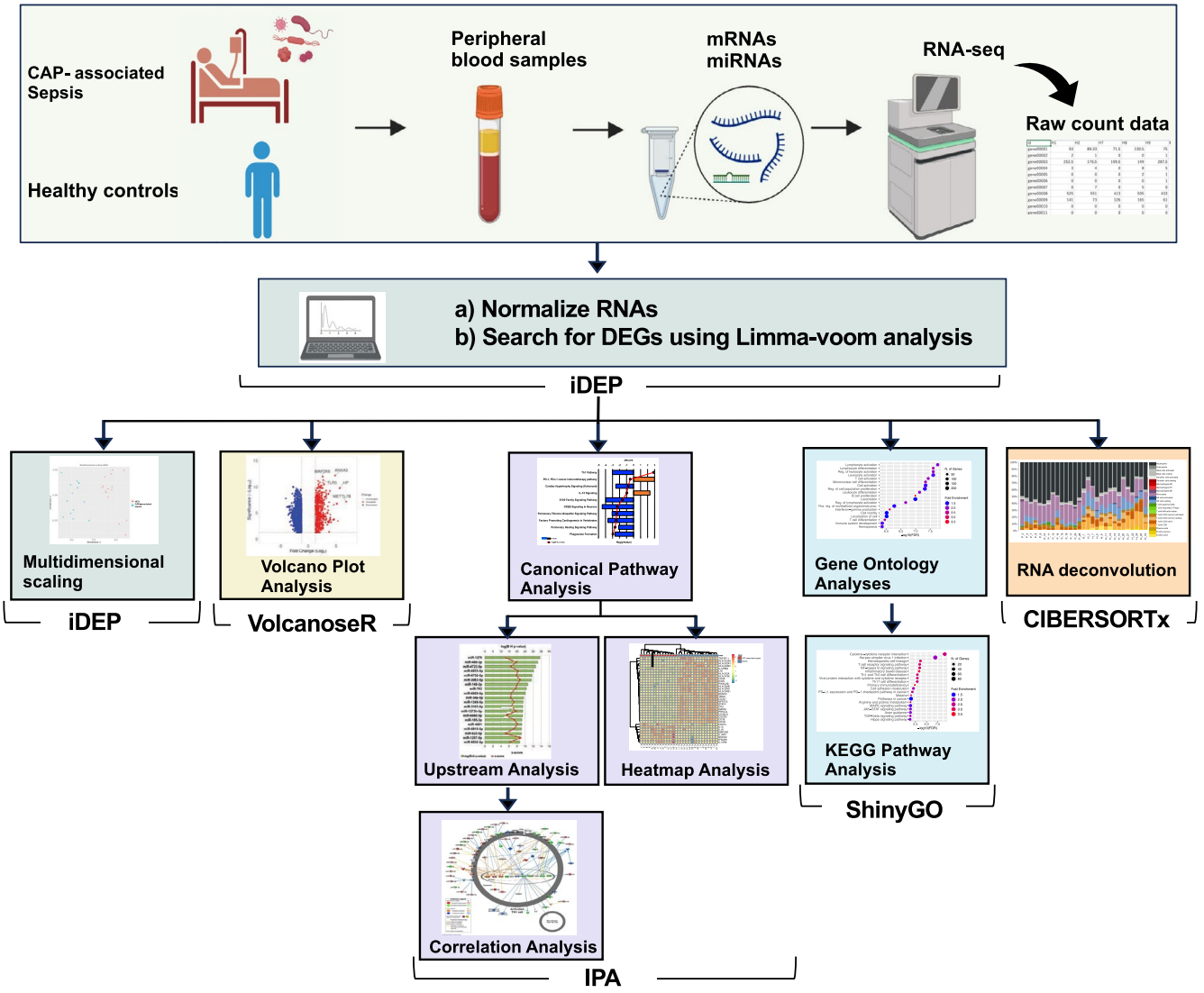

FIGURE 1 Workflow of the study. The raw count data from RNA sequencing was normalized using iDEP; DEGs inversely correlated with miRNAs were included in each analysis. Created with BioRender.com.

this pilot study, we aimed to provide valuable insights for clinical researchers by performing an integrative analysis of mRNA and miRNA profiles in whole blood, identifying key cellular signaling pathways involved in disease progression. Through the discovery of these molecular pathways and their regulatory miRNAs, we explored their potential as therapeutic targets and biomarkers for severe CAP-associated sepsis, ultimately contributing to future advancements in sepsis diagnosis and treatment.

MATERIALS AND METHODS

Study design

This prospective, observational, single-center study included patients with CAP-associated sepsis who were admitted to the Department of Traumatology and Acute Critical Medicine at the Graduate School of Medicine, Osaka University, Japan, from August 2020 to February 2021. The diagnosis of pneumonia was made according to clinical findings, including blood samples and chest computed tomography scans. The definition of pneumonia was an acute illness with cough, at least one new focal chest symptom, fever >4 days, or dyspnea/tachypnea, with at least one of these four conditions present, and without other obvious cause, and was supported by chest radiographic findings of lung shadowing likely to be new.[6] In the elderly, pneumonia is additionally defined by the presence of shadowing on chest radiography that is accompanied by acute clinical illness (unspecified) without other obvious cause.[6] Sepsis was diagnosed in accordance with the Sepsis-3 criteria, which defines sepsis as life-threatening organ dysfunction caused by an abnormal host response to infection, with a baseline change in total Sequential Organ Failure Assessment score of 2 or more points representing organ dysfunction. In addition, septic shock requires vasoconstrictors to maintain a mean blood pressure >65 mmHg despite adequate infusion load, and the blood lactate level is >2 mmol/L or 18 mg/dL.[7] (Figure 2).

Clinical data collection

Data retrieved from electronic medical records of patients included age, sex, body mass index (BMI), Acute Physiology and Chronic Health Evaluation II score, Sequential Organ Failure Assessment score, existing comorbidities (such as hypertension and diabetes), and clinical variables (including

TABLE 1 Patient characteristics.

Baseline characteristic	Patients with CAP-associated sepsis ($n = 14$)	Healthy control subjects ($n = 15$)	p-value
Age (years), median (IQR)	78.0 (67.3–83.5)	55.0 (40.5–59.0)	<0.001
Sex: male (%)	10.0 (71.4)	8.0 (50.0)	0.33
BMI, median (IQR)	21.8 (18.5–25.7)	21.7 (20.5–23.2)	0.911
Comorbidities, *n* (%)			
Diabetes	2.0 (14.3)	1.0 (6.3)	
Hypertension	4.0 (28.6)	2.0 (12.5)	
Hyperlipidemia	1.0 (7.1)	6.0 (37.5)	
Hyperuricemia	0 (0)	1.0 (6.3)	
Chronic heart disease	1.0 (7.1)	1.0 (6.3)	
Chronic lung disease	1.0 (7.1)	0 (0)	
Chronic kidney disease	0 (0)	0 (0)	
Immunocompromised condition	0 (0)	0 (0)	
Malignant neoplasm	5.0 (35.7)	0 (0)	
Mechanical ventilation, *n* (%)	11.0 (78.6)		
Severity of disease on admission			
SOFA score, median (IQR)	5.0 (3.3–5.5)		
PACHEII score, median (IQR)	16.0 (14.0–18.5)		
Disease course			
Length of hospital stay, days, median (IQR)	13.5 (4.3–43.3)		
Hospital mortality, *n* (%)	0 (0)		

Note: p-value was calculated using the Student's t-test. There are missing data on patient and healthy controls (BMI). We used the median imputation method (imputation of missing values using the population median for continuous predictors) or the population mean proportion for categorical predictors derived from the data in which the risk score was originally developed. The p values for age, sex, and BMI were calculated using a one-way analysis of variance.

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation; BMI, body mass index; CAP, community-acquired pneumonia; IQR, interquartile range; SOFA, Sequential Organ Failure Assessment.

the need for intubation and hospital outcome). *p*-values were calculated using the Student's *t*-test.

Library preparation and RNA sequencing

Library preparation and RNA sequencing for mRNA and miRNA were performed as previously described.[8,9] Briefly, blood samples were collected on the day of admission from patients and healthy controls (HCs), and RNA was isolated from leukocytes using the PAXgene Blood RNA System.

Statistical analysis

The workflow of this study is illustrated in Figure 1. Raw mRNA count data were normalized as previously described.[8,9] Limma–Voom analysis[10] differential expression between patients with CAP-associated sepsis and HCs. Deconvolution analysis was performed using the web tool CIBERSORTx (https://cibersortx.stanford.edu/), a machine learning algorithm.[11] RNA sequencing data were processed into a gene expression matrix and analyzed using CIBERSORTx with the LM22 signature matrix, which identifies 22 human immune cell types. The output provided the relative abundance of these cell subtypes per sample.

Among the immune cell subtypes, changes in the proportions of neutrophils, lymphocytes, and monocytes between patients with CAP-associated sepsis and HCs were analyzed.[11]

Volcano plots were created to visualize and identify key differentially expressed genes, using a $\log_2$ fold change threshold >1.2 and a false discovery rate < 0.05. Ingenuity Pathway Analysis (IPA 2022 fall, QIAGEN Inc.,https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) was used to predict the activation or inhibition of pathways as previously described.[8,9] Adjusted *p* values were computed using the Benjamini–Hochberg

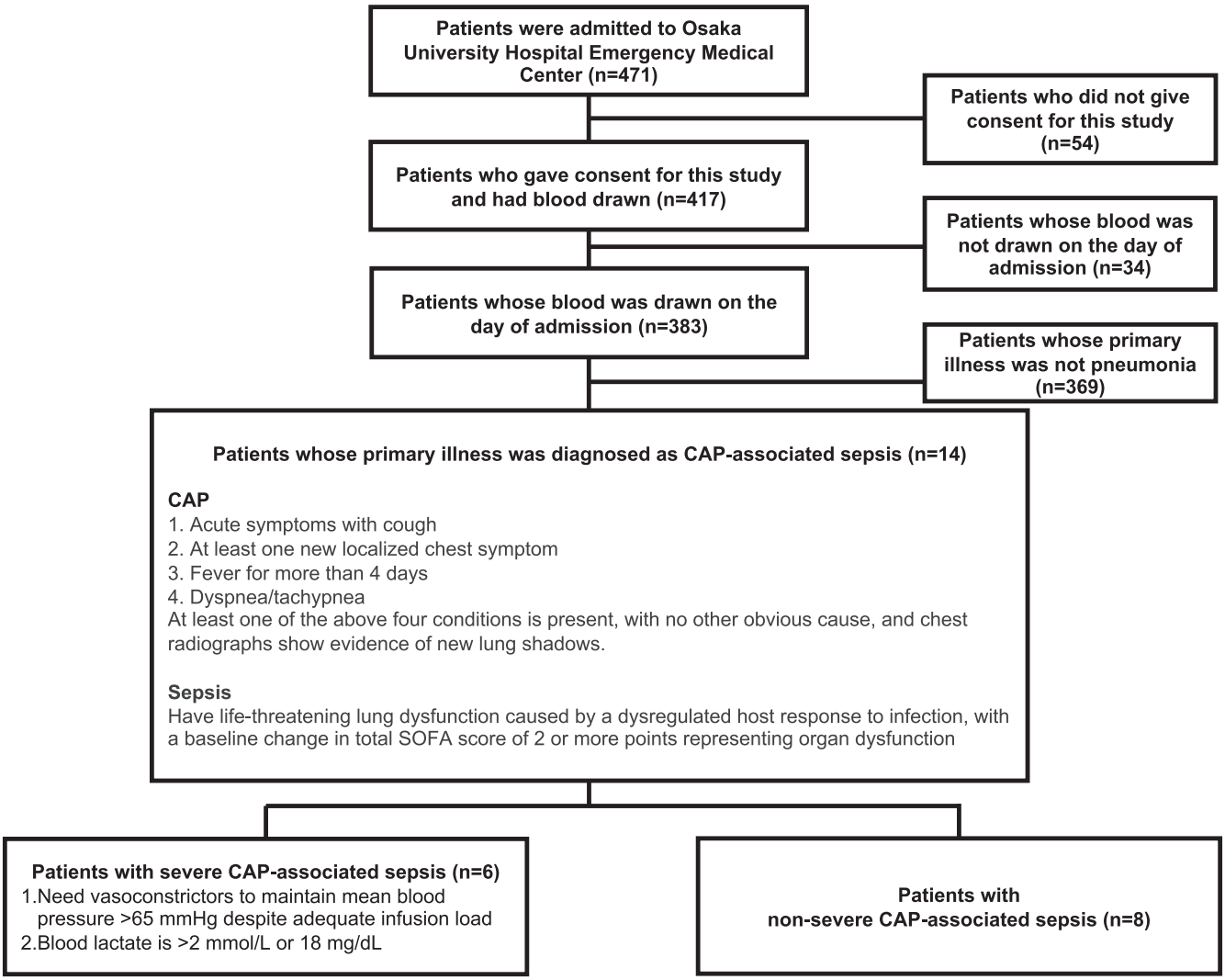

FIGURE 2 Patient flowchart and definitions of community-acquired pneumonia and sepsis.

method,[12] with pathways activated at z-scores >2 and $p < 0.05$. Upstream regulator analyses were performed using RNA's expression. Gene set enrichment analysis was performed to validate the results of Canonical pathway analysis (CPA) using IPA. We used two types of analysis—Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses using the web tool ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/).[13] Furthermore, to assess miRNA gene expression variations between severe and non-severe CAP groups, the presence or absence of septic shock was set as the outcome, and the Wilcoxon signed-rank test[14] was performed.

RESULTS

Patient characteristics

The study involved 14 critically ill patients and 15 HCs. The median ages of the patients and HCs were 78 and 55 years, respectively, with BMIs of 22.8 and 21.7 kg/m^2, respectively. No significant differences were observed for any variable except age between the two groups (Table 1). All patients were treated at an advanced critical care center. The patient flowchart is presented in Figure 2. All patients tested negative for coronavirus disease 2019. Five patients had a history of malignant neoplasm, including two with breast cancer, one with thyroid cancer, one with right renal cell carcinoma, and one with prolactinoma. All five patients had undergone surgery but had not received chemotherapy with immune checkpoint inhibitors. Details regarding the causative organisms of pneumonia are presented in Figure S1.

Statistical analysis of mRNAs and miRNAs

Multidimensional scaling demonstrated that the expression levels of mRNA, miRNAs, and mRNAs related to miRNAs could be used to distinguish between patient and HCs groups (Figure 3A). Volcano plots revealed that 13,916 mRNAs, 1797 miRNAs, and 1920 mRNAs related to miRNAs were differentially expressed and included in further analysis. The numbers of genes showing upregulated versus downregulated expression variations (FDR < 0.05, $|\log_2 FC| > 1.2$) were 1209:1461 for mRNAs, 51:21 for miRNAs, and 646:1274 for mRNAs related to miRNAs (Figure 3B). Significant differences were observed in the proportions of neutrophils, monocytes, and lymphocytes between patients with CAP-associated sepsis and HCs (Figure S2).

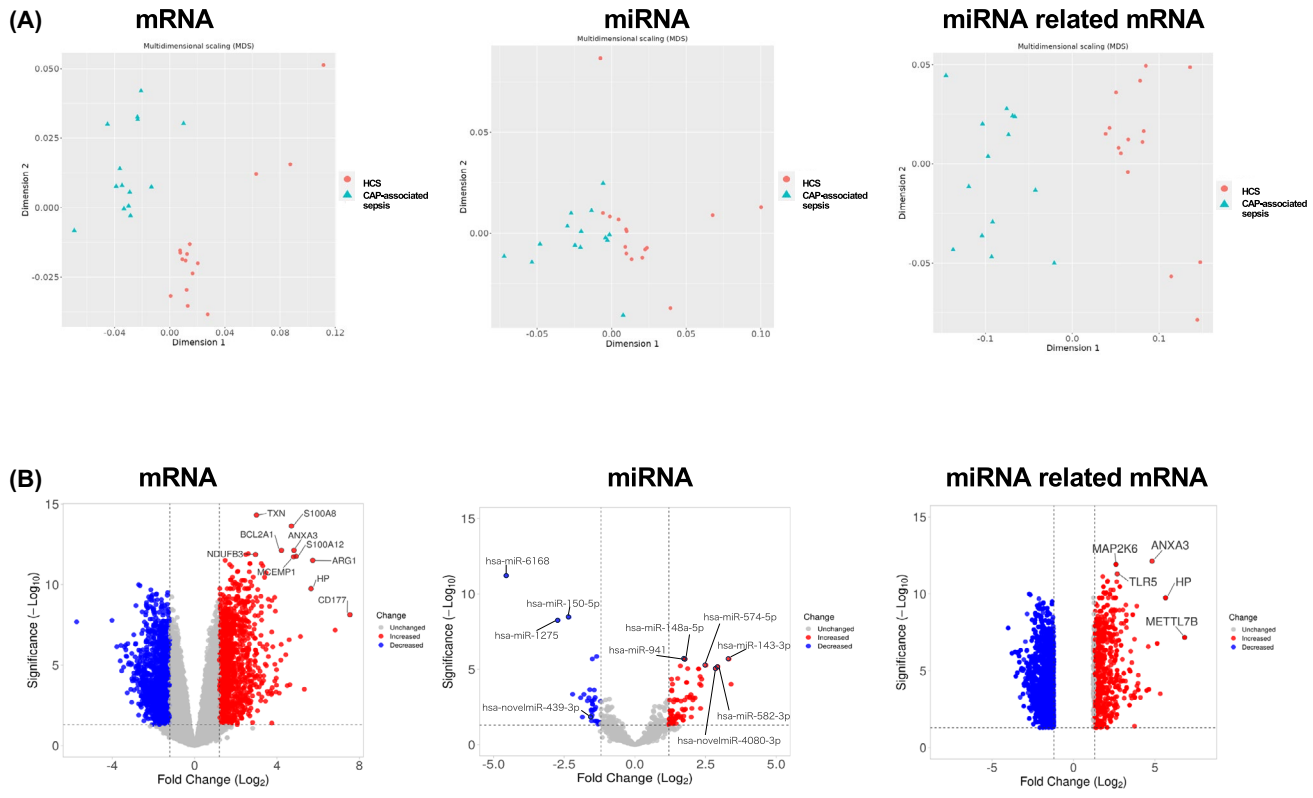

FIGURE 3 Expression variation and multidimensional scaling analysis of total mRNAs, miRNAs, and mRNAs related to miRNAs. (A) Multidimensional scaling analysis of total mRNAs, miRNAs, and mRNAs related to miRNAs. (B) Expression variation analysis representing mRNAs and miRNAs expression specifically expressed in patients with pneumonia compared with that in healthy controls. Significant differential expression of RNA is shown. Vertical dotted lines represent $|\log_2 FC| > 1.2$; horizontal dotted line represents FDR < 0.05; red dots: Increased expression; blue dots: Decreased expression.

Canonical pathway and pathway analyses

CPA predicted mRNA activation of 30 pathways and inhibition of 33 pathways (the top 10 pathways are shown in Figure 4A). Among the activation pathways, the programmed death-1 (PD-1) and programmed cell death ligand 1 (PD-L1) cancer immunotherapy signaling pathways showed the most significant p value (BH adjusted $p = 5.2E\text{-}01$, z-score = 3.266). Twenty-eight factors were involved in PD-1 and PD-L1 cancer immunotherapy signaling in a heatmap of mRNA (Figure 4B). The variation in each of these factors was generally divided between the pneumonia patients and the HCS. Among the inhibited pathways, the T helper 1 (Th1) signaling pathway showed the most significant p value (BH adjusted $p = 7.7E\text{-}01$, z-score = –2.694). Thirty-seven factors were involved in the Th1 signal, and the variation of each was generally divided between the pneumonia patients and the HCS (Figure 4C). The Th1 signal showed a decrease in key factor TBX21(also known as T-bet), whereas the signal itself was decreased (z-score ≤ –2).

CPA of mRNAs related to miRNAs helped identify the activation of six activated pathways and the inhibition of 52 inhibited pathways (the top 10 pathways are presented in Figure 4D). Among the activated pathways, PD-1 and PD-L1cancer immunotherapy pathways had the most significant p value (Benjamini–Hochberg-adjusted $p = 6.6E\text{-}01$, z-score = 2.985). Among the inhibited pathways, the Th1 pathway, involving 34 factors, had the most significant p value (Benjamini–Hochberg-adjusted $p = 8.9E\text{-}01$, z-score = –3.024) (Figure 4E,F). The expression of *TBX21* was downregulated in these patients. Gene variability between

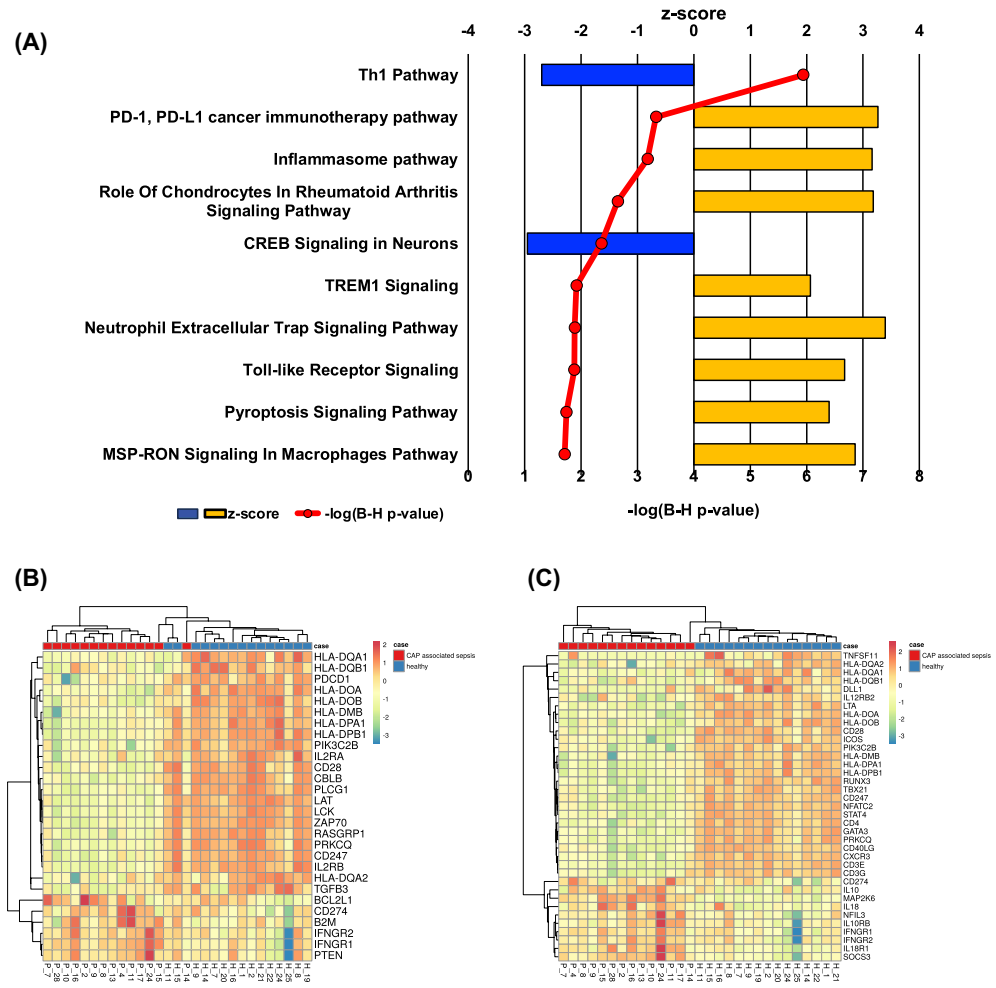

FIGURE 4 Canonical pathway analysis (CPA) and upstream analysis. (A) CPA of mRNAs. Top 8 activated and 2 inactivated signaling pathways identified using Ingenuity Pathway Analysis. (B) Heatmap of genes involved in PD-1 and PD-L1 cancer immunotherapy signaling pathways in mRNAs calculated using RNA-Seq. (C) Gene expression involved in the Th1 signaling pathway in mRNAs calculated using RNA-Seq. (D) CPA of mRNAs related to miRNAs. Top 2 activated and top 8 inactivated signaling pathways identified using Ingenuity Pathway Analysis. (E) Gene expression involved in the PD-1 and PD-L1 cancer immunotherapy signaling pathways in mRNAs related to miRNAs calculated using RNA-Seq. (F) Heat map of genes involved in the Th1 signaling pathway in mRNAs related to miRNAs calculated using RNA-Seq. (G) Top 20 activated upstream regulators in mRNAs. (H) Top 20 inactivated upstream regulators in mRNA. (I) Top 20 activated upstream regulators in the mRNAs related to miRNAs. (J) Top 20 inactivated upstream regulators in the mRNAs related to miRNAs.

patients and HCS was generally divided. Furthermore, suppression of CREB signaling in neurons and upregulation of the anti-inflammatory cytokine IL-10 pathway were observed (Figure 4E,F). Compared with the results of CPA by IPA, GO analysis predicted activation of cellular macromolecule localization and some protein pathways in mRNAs (Figure S3A), as well as activation of lymphocyte activation in mss related to miRNAs (Figure S3B). KEGG pathway analysis helped predict the activation of metabolic pathways in mRNA (Figure S3C) and the activation of PD-1 and PD-L1-related pathways in mRNAs related to miRNAs (Figure S3D).

Upstream analysis of mRNAs revealed activated to inhibited gene expression values of 937:131 ($p < 0.05$). The top 3 among the top 20 activators were *TNF*, *IL1B*, and *ID3* ($p < 0.05$; Figure 4G), whereas the top 3 inhibitors were miR-6882-5p, *SOX4*, and *APOE* (Figure 4H). In the upstream analysis of mRNAs related to miRNAs, activated to inhibited gene expression values were 1620:39 ($p < 0.05$). The top 3 among the top 20 activators were miR-1275, miR-486-3, and miR-4723-5p (Figure 4I), and the top inhibitors were *IL7R*, *N6AMT1*, and *PAX5* (Figure 4J).

Comparison of miRNA gene expression levels between patients with severe and non-severe CAP-associated sepsis

The Wilcoxon signed-rank test between the severe CAP-associated sepsis group ($n = 6$ with septic shock) and the non-severe CAP-associated sepsis group ($n = 8$ without septic shock) showed significant differences in the expression of miR-148a-5p, miR-148b-5p, miR-184, miR-192-5p, miR-199a-5p, miR-221-5p, miR-30a-3p, miR-374b-5p, miR-3909, miR-4746-5p, miR-548ae-3p, and miR-6503-3p. These two groups exhibited no significant differences in age, sex, or BMI. These results are presented in Figure S4, while a schematic representation of the putative PD-1/PD-L1 cancer immunotherapy pathways and their relationship with Th1 cells is depicted in Figure 5.

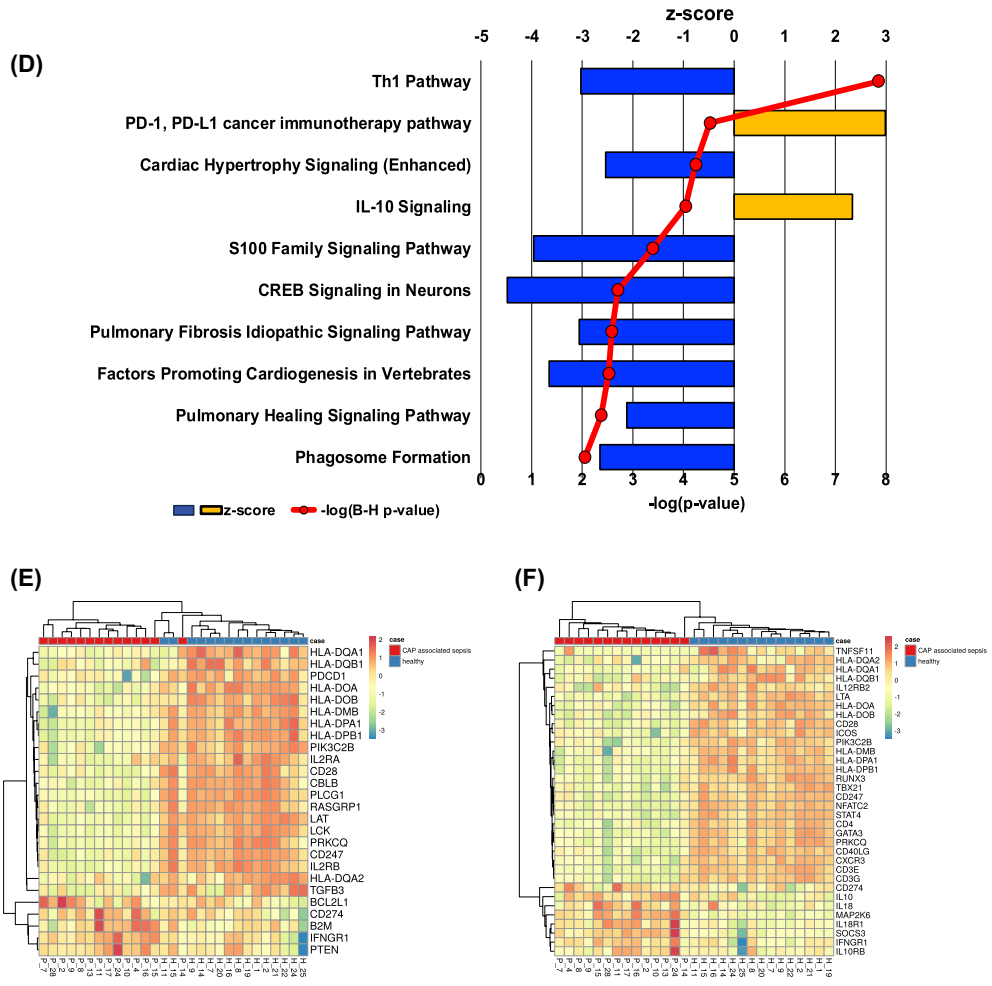

FIGURE 4 (Continued)

DISCUSSION

miRNAs reportedly serve as biomarkers for inflammatory diseases.[15] Therefore, we investigated the relationship between cellular signals and miRNAs in the acute phase of CAP-associated sepsis using mRNA–miRNA integration analysis to characterize the disease pathogenesis. Five patients had a history of malignancy. All had undergone surgical treatment, completed their malignancy-related treatments, and had not received chemotherapy involving immune checkpoint inhibitors. While it is not possible to definitively rule out any effects of their malignancy on this study due to the lack of RNA sequence data specific to their malignancies, multidimensional scaling analysis (Figure 3) and RNA deconvolution (Figure S2) revealed no significant differences between these five patients and the others. Therefore, we included these patients in the analysis alongside the rest of the cohort.

Pathogen persistence can lead to dysregulation of the host immune response, resulting in sepsis owing to inadequate immune responses.[16] In the current study, these dysregulated responses were associated with suppression of the Th1 pathway, CREB signaling in neurons, and upregulation of the anti-inflammatory IL-10 pathway.[17] Several specific transcriptional pathways have been implicated in T-cell exhaustion. For example, Blimp-1, a transcriptional repressor, is upregulated during CD8+ T-cell exhaustion, driving terminal differentiation and inhibitory receptor expression, including PD-1. Combining PD-1 inhibitors with IL-10 blockers or therapeutic vaccines may enhance immunity in chronic infections.[18] In this study, the PD-1/PD-L1 and IL-10 signaling pathways were also upregulated.

PD-1 plays a vital role in suppressing immune responses and promoting self-tolerance by regulating T-cell activity and inhibiting regulatory T-cell apoptosis.[19] Notably, activating anti-tumor immunity by inhibiting immune checkpoints

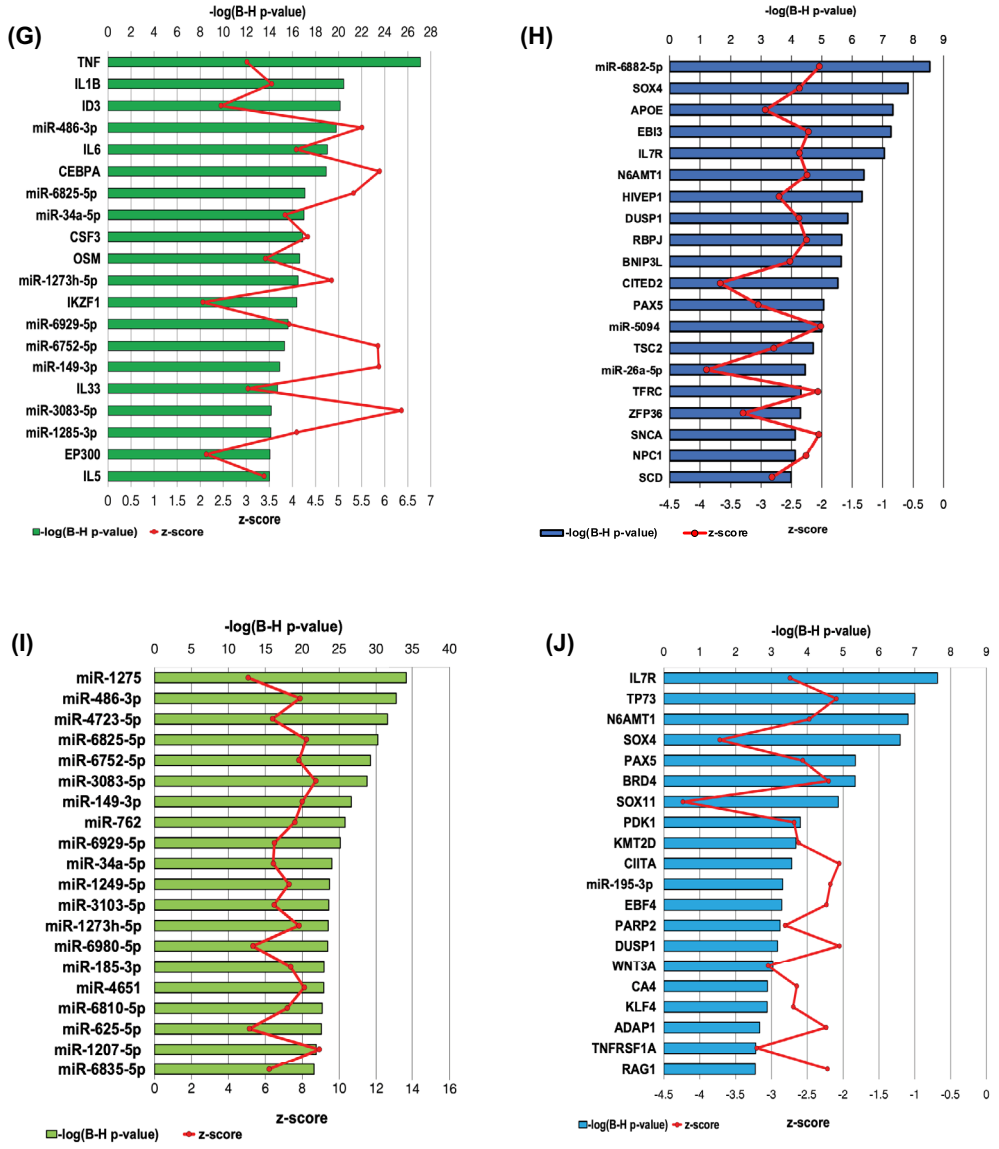

FIGURE 4 (Continued)

in the PD-1/PD-L1 pathway is considered a promising approach for cancer therapy.[20] In contrast, PD-1 on T cells and PD-L1 on monocytes are upregulated in patients with severe sepsis and septic shock. Hence, PD-L1 signaling might play a vital role in sepsis-induced immunosuppression by acting on exhausted T cells and releasing immunosuppressive molecules.[16,19]

Postmortem studies of patients who died of sepsis showed T-cell depletion and signs of T-cell exhaustion.[21] Sepsis induces an anti-inflammatory state through altered immune cell positioning and suppressed Th1 and monocyte responses, reducing proinflammatory cytokines like TNF, IL-1β, and IL-6.[16] Intense anti-inflammatory responses may induce a state of immunosuppression in patients with sepsis,[17] consistent with our findings. Upstream regulator analysis in IPA helped identify mRNA expression of *TNF*, which is involved in PD-1 and PD-L1 cancer immunotherapy signaling, and IL-6, which is involved in Th1 signaling(Figure 4G–J). These findings suggest that activation of these signals is important

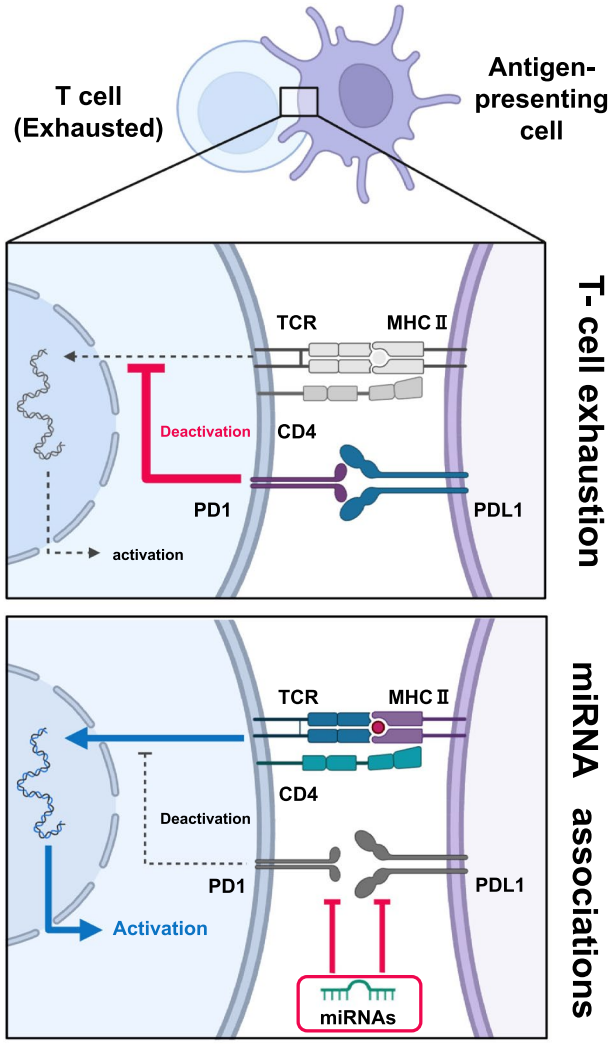

FIGURE 5 Schematic representation of putative PD-1 and PD-L1 cancer immunotherapy pathways and their association with Th1 cells. Prepared using BioRender.com.

in the pathogenesis of CAP-associated sepsis. This observation is consistent with reports that PD-1 activation is accompanied by suppression of cellular functions, especially those of T cells, inactivation of the Th1 pathway, and strong T-cell exhaustion during the acute phase of CAP.

Diverse associations have been reported between infections and human leukocyte antigens (HLAs); in sepsis, an association with HLA-DQB1 has been suggested.[22] T-bet and GATA3(a transcription factor encoded by *GATA3* in humans), specific transcription factors for Th1 and Th2 cells, respectively, are significantly downregulated in patients with sepsis compared with those in HCs.[23] Similarly, in the current study, the expression of T-bet and GATA3 in patients with CAP-associated sepsis was significantly suppressed compared with that in HCs (Figure 4C,F).

The human genome comprises 3 billion base pairs, with only 1%–2% coding for proteins; the majority consists of noncoding regions like repetitive sequences and introns.[24] ncRNAs are important regulators of gene expression networks that help regulate nuclear structure and transcription[25] In this study, the expression of 12 miRNAs was predominantly variable. The upregulation of hsa-miR-199a-5p is associated with neoplastic lesion progression, such as lung cancer and oral squamous cell carcinoma.[26] These findings suggest that miR-199a-5p is involved in severe inflammation. Conversely, miR-150 is significantly downregulated in most cases of acute myeloid leukemia and colorectal cancer.[27] Our findings also revealed downregulation of miR-150-3p, implying lymphocyte depletion in severe inflammation. Therefore, the 12 miRNAs identified in this study and implicated in the pathogenesis of severe CAP may be potential targets for developing novel therapeutics and biomarkers. Collectively, our findings may help elucidate the pathogenesis of CAP-associated sepsis. This study provides foundational insights into the molecular mechanisms underlying severe CAP-associated sepsis by identifying key cellular signaling pathways and miRNAs involved in its pathogenesis. Our findings may provide insight into the complex interplay between gene regulation and immune response in sepsis. These results contribute to the growing body of sepsis research findings and highlight specific miRNAs and pathways that may serve as novel therapeutic targets or biomarkers. Future studies with larger cohorts and functional validation are necessary to elucidate these molecular mechanisms and their potential clinical applications.

LIMITATION

This study has several limitations because this is a pilot study. First, this study was observed in a single center, so we did not analyze enough numbers of patients with CAP associated sepsis. Our results will need to be confirmed in a larger prospective study in the future. Second, pneumonia cases were not classified based on the bacteria causing the inflammation. Additionally, whole blood samples may have contained many components and factors that could

have interfered with the analysis. Finally, heterogeneity was observed between patients with sepsis and HCs; HCs differed significantly from patients with sepsis in some aspects, including age.

CONCLUSION

Collectively, our findings indicate that miRNAs regulate the Th1, PD-1, and PD-L1 cancer immunotherapy pathways through mRNA interference. These findings suggest that T-cell exhaustion may occur during the acute phase of CAP-associated sepsis.

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CONFLICT OF INTEREST STATEMENT

Hiroshi Ogura is an Editorial Board member of AMS Journal and a co-author of this article. To minimize bias, he was excluded from all editorial decision-making related to the acceptance of this article for publication. Jun Oda is the Editor-in-Chief of the journal. He was excluded from the peer review process and all editorial decisions related to the acceptance and publication of this article. Peer review was handled independently by the AMS Journal editorial office and deputy EiC as editor to minimize bias. All other authors state that we have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The raw data of this study can be accessed at Gene Expression Omnibus under accession number GSE228542.

ETHICS STATEMENT

This study was performed in compliance with the principles of the Declaration of Helsinki and was approved by the institutional review board of Osaka University Hospital (approval no.: 885 [Osaka University Critical Care Consortium Novel Omix Project; Occonomix Project]). Informed consent was obtained from the patients, their relatives, and healthy volunteers for the collection of blood samples. Anonymized information was used in the study, and no identifiable image was included in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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