Gene expression profile analysis of ventilator-associated pneumonia

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Abstract. Based on the gene expression profile of patients with ventilator-associated pneumonia (VAP) and patients not affected by the disease, the present study aimed to enhance the current understanding of VAP development using bioinformatics methods. The expression profile GSE30385 was downloaded from the Gene Expression Omnibus database. The Linear Models for Microarray Data package in R language was used to screen and identify differentially expressed genes (DEGs), which were grouped as up- and down-regulated genes. The up- and downregulated genes were functionally enriched using the Database for Annotation, Visualization and Integrated Discovery system and then annotated according to TRANSFAC, Tumor Suppressor Gene and Tumor Associated Gene databases. Subsequently, the protein-protein interaction (PPI) network was constructed, followed by module analysis using CFinder software. A total of 69 DEGs, including 33 up- and 36 downregulated genes were screened out in patients with VAP. Upregulated genes were mainly enriched in functions and pathways associated with the immune response (including the genes *ELANE* and *LTF*) and the mitogen-activated protein kinase (MAPK) signaling pathway (including *MAPK14*). The PPI network comprised 64 PPI pairs and 44 nodes. The top two modules were enriched in different pathways, including the MAPK signaling pathway. Genes including *ELANE*, *LTF* and *MAPK14* may have important roles in the development of VAP via altering the immune response and the MAPK signaling pathway.

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Introduction

Ventilator-associated pneumonia (VAP), the most common type of hospital-acquired pneumonia (HAP), is defined as bacterial pneumonia that develops in patients who have received mechanical ventilation for >48 h (1). VAP occurs in 6-52% of mechanically ventilated patients and in certain specific settings, the incidence is up to 76% (2). The incidence of VAP varies depending on the type of population studied, the intensity of preventive measures implemented and the presence or absence of risk factors. With an estimated attributable mortality of 9%, VAP usually increases the time of mechanical ventilation and time spent in the intensive care unit (ICU) by >10 days and its treatment accounts for at least 50% of antibiotics prescribed in ICUs, producing a huge cost (3). The expected burden of VAP in the coming years may increase with the increasing age of the population, intensification of care and the growing prevalence of severe underlying diseases in ICU patients (4). As the diagnosis as well as the treatment of VAP remain challenging, studies are required to gain a comprehensive understanding of VAP.

A number of previous studies have shown that up- or down-regulation of the expression of certain genes is closely associated with the development of infection. For instance, a study on the polymorphism of tumor necrosis factor (TNF)-α reported that overexpression of TNF-α is associated with a 2.1-13-fold increase in the incidence of severe sepsis from all causes, including pneumonia (5). *PI3*, which encodes an elastase-specific inhibitor with antimicrobial peptide activity, has proven to promote early clearance of *Pseudomonas aeruginosa* by activating macrophages and recruiting neutrophils (6). *PIK3R3*, which encodes the protein of phosphoinositide 3-kinase regulatory sub-unit gamma, was shown to be predominately expressed in immune cells and to be involved in chemoattractant-induced cell migration (7). None of these genes alone, however, is sufficient enough to answer the fundamental question why certain patients develop VAP, while other similar patients do not.

As a genome-wide screening approach, microarray analysis may be effective for identifying novel genetic factors or gene expression profiles associated with the development of

infection in VAP. With the goal to construct a model for the prediction of susceptibility to VAP in critically-injured trauma patients, Swanson *et al* (8) analyzed the gene expression profiles of patients who developed VAP and those who were never affected. By comparing the differentially expressed genes (DEGs) between the two groups, a logistic regression model was constructed, which comprised five genes (*PIK3R3, ATP2A1, PI3, ADAM8* and *HCN4*) and was able to accurately categorize 95% of patients that developed VAP.

The present study identified DEGs between patients with VAP and those without VAP based on the expression profiling data provided by Swanson *et al* (8) and then performed functional annotation and enrichment analyses in order to elucidate the potential molecular mechanisms of VAP. In addition, a protein-protein interaction (PPI) network was constructed and the functional modules in this network were analyzed. The novel view on VAP provided by the present study indicates that VAP requires further study.

Materials and methods

Derivation of genetic data. The gene expression profile dataset GSE30385 deposited by Swanson *et al* (8) was downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo), a public functional genomics data repository. The annotation platform was the GPL201 (HG-Focus) Affymetrix Human HG-Focus Target Array platform (Affymetrix, Inc., Santa Clara, CA, USA) updated on May 10th, 2013. A total of 20 samples from critically ill trauma patients were available, including 10 patients who developed ventilator-associated pneumonia (VAP group) and 10 who did not (NoVAP group).

Data processing. The Robust Multichip Average method of the R/Bioconductor Affy package (http://www.bioconductor.org/; Affymetrix, Inc.) was used to pre-process the downloaded raw data via background adjustment, quintile normalization and summarization (9). Using the R/Bioconductor software v 3.1 (http://www.bioconductor.org/), probe serial numbers in the matrix were transformed into gene names. As certain different probes can be mapped to the same gene, the average probe expression value was calculated as the final expression value of the corresponding gene.

Screening of DEGs. To screen DEGs between the VAP group and the NoVAP group, the Linear Models for Microarray Data (Limma) package in R/Bioconductor software 3.1 (http://www.bioconductor.org/packages/release/bioc/html/limma.html) was used (10). The Benjamini-Hochberg false discovery rate (FDR) was used to adjust the raw P-value in the multiple testing (11,12). DEGs were screened with cutoff values of FDR<0.05 and |log (fold change)|≥1.5 (13,14). The eligible DEGs were grouped into upregulated and downregulated genes. To guarantee that the identified DEGs represent the two types of specimen, cluster analysis of DEGs was also performed using the Pheatmap package of R (http://cran.r-project.org/web/packages/pheatmap/index.html).

Functional annotation and pathway enrichment analysis. The Database for Annotation Visualization and Integrated Discovery (DAVID) provides a comprehensive set of functional annotation tools to understand the biological meaning of large lists of genes by using a hypergeometric distribution algorithm (15). To functionally annotate DEGs identified between the VAP group and the NoVAP group, DAVID was used to analyze the significantly enriched Gene Ontology (GO) terms (16) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (17) for upregulated and down-regulated genes, respectively (adjusted P-value <0.05).

Computational identification of transcription factors (TF), tumor suppressor genes (TSGs) and oncogenes. TRANSFAC version 7.0 (http://www.gene-regulation.com/pub/databases.html), which was initially a data collection, has evolved into the basis for a complex platform for the description and analysis of gene regulatory events and networks, including TFs as well as their DNA-binding sites and profiles (18). The TSGene database was designed by integrating TSGs with large-scale experimental evidence to offer a comprehensive resource for further investigation of TSGs and their molecular mechanisms in cancer (19). The tumor-associated gene (TAG) database was developed by integrating information from well-characterized oncogenes and tumor suppressor genes to facilitate cancer research (20). Based on these three databases, DEGs identified between the VAP group and the NoVAP group were analyzed for the screening of TFs, TSGs and TAGs.

Protein-protein interaction (PPI) network construction. The Search Tool for the Retrieval of Interacting Genes (STRING version 1.0; http://string-db.org/) is an online database which includes experimental as well as predicted interaction information and comprises >1,100 completely sequenced organisms (21). The DEGs identified above were directly mapped to the STRING database for acquiring significant PPI pairs from a range of sources, including data from experimental studies and data retrieved by text mining and homology searches. PPI pairs with the combined score of ≥0.4 were retained for the construction of the PPI network.

Functional module analysis. CFinder (http://www.cfinder.org/) is a fast program for locating and visualizing overlapping, densely interconnected groups of nodes (defined as modules) in undirected graphs, and allows for easy navigation between the original graph and the network of these groups (22). Using the default parameter of k=3, modules were screened from the PPI network constructed as described above using CFinder. The top two sub-modules identified by modularity analysis were then selected for later functional enrichment by DAVID (FDR<0.05).

Results

Identification of DEGs. With a FDR<0.05 and |log (fold change)|≥1.5, 69 DEGs between the VAP group and the NoVAP group were obtained. Of these DEGs, 33 (47.83%) were upregulated and 36 (52.17%) were downregulated in the VAP group compared with the NoVAP group. The results of the clustering analysis of the DEGs are shown in Fig. 1.

Functional annotation and pathway enrichment analysis. To identify the functions of these DEGs, all of the up- and downregulated genes were mapped to terms of the GO data-

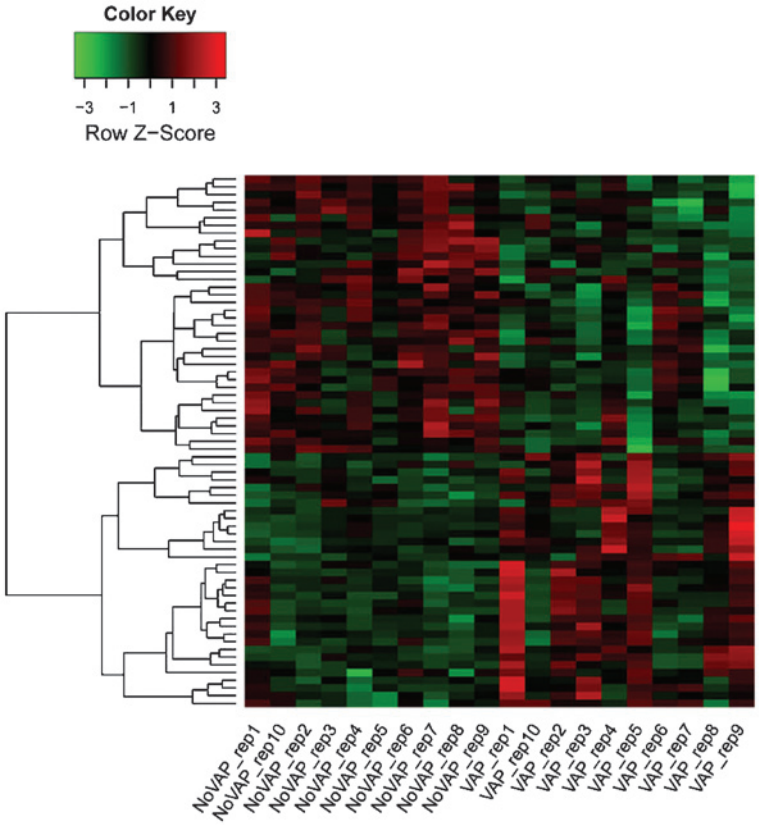

Figure 1. Dendrogram of differentially expressed genes identified by cluster analysis.

base, which consists of three ontologies, namely biological processes (BP), cellular components (CC) and molecular function (MF). In the three categories of the GO terms, 'immune system processes', 'immune response', 'kinase activity' were dominant for the upregulated genes, while 'response to stress', 'programmed cell death' and 'peptidase inhibitor activity' were dominant among the downregulated genes (Table I).

KEGG was used to further identify the altered biological functions arising from these DEGs. The upregulated genes were mainly enriched in ten pathways, including the neurotrophin signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway and the nucleotide-binding oligomerization domain (NOD)-like receptor signaling pathway, while the downregulated genes were enriched in nine pathways, which included complement and coagulation cascades as well as pathways in cancer and ribosomes (Table II).

Identification of TFs, TSGs and oncogenes. According to the TRANSFAC, TSGene and TAG databases, a total of four TFs, two oncogenes, four TSGs and two other TAGs were further screened from the DEGs preliminarily identified between the VAP group and the NoVAP group (Table III).

PPI construction. The PPI network contained 64 PPI pairs and 44 nodes (20 upregulated genes and 24 downregulated genes in the VAP group) (Fig. 2). The five node proteins with a connection degree of >5 were ELANE (connection degree, 10), PTGS2 (connection degree, 9), MAPK14 (connection degree, 8), LTF (connection degree, 7) and MMP8 (connection degree, 6).

Functional module analysis. High aggregation, which reflects the high modularization of a gene network, is an important characteristic of biological networks. To distinguish the modules with specific functions and different sizes, the constructed network was usually divided into relatively independent sub-modules prior to analysis. By using CFinder, three models were obtained using the default parameter of k=3 and the top two modules are shown in Fig. 3.

The significantly enriched GO terms and KEGG pathways for Module 1 and Module 2 are displayed in Tables IV and V, respectively. Genes in Module 1 were mainly classified into the GO terms of multi-organism processes, extracellular region and serine-type endopeptidase activity, while genes in Module 2 were mainly classified into the GO terms negative regulation of biological processes, nucleus and protein kinase activity. Genes in Module 2 were enriched in 12 pathways, including the MAPK, p53 and VEGF signaling pathways, while no significant pathway was identified for genes in Module 1 at FDR<0.05.

Discussion

Due to its high prevalence and mortality, there is an urgent requirement to investigate the pathogenic mechanism of VAP. The present study analyzed the gene expression profiles of patients who developed VAP and those who were not affected in order to identify possible functions and signaling pathways

Table I. Classification of differentially expressed genes in ventilator-associated pneumonia according to GO terms with P<0.05.

Gene category	GO ID	Function	Count	P-value
Upregulated genes				
BP	GO:0009617	Response to bacterium	9	6.66×10^{-8}
	GO:0006955	Immune response	14	2.12×10^{-7}
	GO:0051707	Response to other organism	9	5.28×10^{-6}
	GO:0009607	Response to biotic stimulus	9	7.59×10^{-6}
	GO:0002376	Immune system process	15	9.69×10^{-6}
CC	GO:0005576	Extracellular region	13	0.0001
	GO:0071682	Endocytic vesicle lumen	2	0.0004
	GO:0030139	Endocytic vesicle	4	0.0005
	GO:0005615	Extracellular space	6	0.0052
	GO:0045335	Phagocytic vesicle	2	0.0076
MF	GO:0004908	Interleukin-1 receptor activity	2	8.24×10^{-5}
	GO:0035251	UDP-glucosyltransferase activity	2	0.0001
	GO:0046527	Glucosyltransferase activity	2	0.0002
	GO:0004708	Kinase activity	2	0.0005
	GO:0004712	Protein serine/threonine/tyrosine kinase activity	2	0.0023
Downregulated genes				
BP	GO:0050873	Brown fat cell differentiation	3	5.08×10^{-5}
	GO:0006950	Response to stress	18	0.0001
	GO:0050790	Regulation of catalytic activity	12	0.0001
	GO:0006915	Apoptotic process	12	0.0002
	GO:0012501	Programmed cell death	12	0.0002
CC	GO:0044445	Cytosolic part	4	0.0005
	GO:0022626	Cytosolic ribosome	3	0.0009
	GO:0044421	Extracellular region part	8	0.0019
	GO:0044391	Ribosomal subunit	3	0.0026
	GO:0005829	Cytosol	12	0.0036
MF	GO:0042277	Peptide binding	4	0.0004
	GO:0033218	Amide binding	4	0.0005
	GO:0004866	Endopeptidase inhibitor activity	4	0.0006
	GO:0061135	Endopeptidase regulator activity	4	0.0006
	GO:0030414	Peptidase inhibitor activity	4	0.0006

GO, gene ontology; BP, biological process; CC, cellular components; MF, molecular function.

Figure 2. Protein-protein interaction networks of differentially expressed genes.

Table II. Enrichment of KEGG pathways in ventilator-associated pneumonia with P<0.05.

Gene category	KEGG ID	Function	Count	P-value
Upregulated genes	4722	Neurotrophin signaling pathway	3	0.0064
	4010	Mitogen-activated protein kinase signaling	4	0.0078
	5014	Amyotrophic lateral sclerosis	2	0.0112
	4621	Nucleotide-binding oligomerization domain receptor signaling pathway	2	0.0133
	4664	Fc epsilon RI signaling pathway	2	0.0238
	4640	Hematopoietic cell lineage	2	0.0291
	4912	Gonadotropin-releasing hormone signaling	2	0.0375
	4620	Toll-like receptor signaling pathway	2	0.0382
	5146	Amoebiasis	2	0.0410
	4060	Cytokine-cytokine receptor interaction	3	0.0449
Downregulated genes	4610	Complement and coagulation cascades	4	0.0001
	3010	Ribosome	3	0.0053
	5150	*Staphylococcus aureus* infection	2	0.0192
	5131	Shigellosis	2	0.0234
	5140	Leishmaniasis	2	0.0318
	5220	Chronic myeloid leukemia	2	0.0326
	5200	Pathways in cancer	4	0.0358
	5222	Small cell lung cancer	2	0.0431
	4012	ErbB signaling pathway	2	0.0449

KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table III. Annotation for TFs, TSGs, oncogenes and other TAGs in ventilator-associated pneumonia.

Gene category	TFs	Oncogenes	TSGs	Other TAGs
Upregulated genes	KLF7	CD24	LTF	NA
Downregulated genes	EGR3, FOSB, NCOR2	CRK	BTG2, CDKN1A, THBD	RGS2, CTSZ

TF, transcription factor; TSG, tumor suppressor gene; TAG, tumor-associated gene.

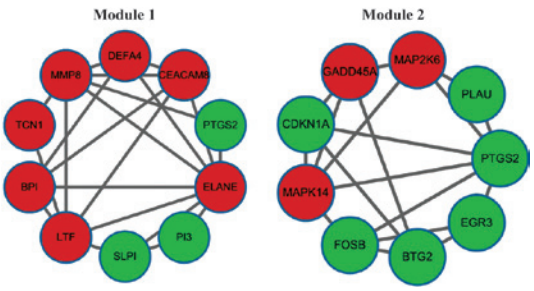

Figure 3. Top two modules in the protein-protein interaction network.

of DEGs between the two groups. After the construction of a PPI network, sub-modules in the network were mined and the functions of the top two modules were annotated.

In the present study, a total of 69 DEGs were identified between patients with VAP and those not affected. This result differs from that of Swanson *et al* (8), who provided the gene expression profile and identified 810 DEGs between the two groups. The inconsistency between the present study and that by Swanson *et al* (8) is likely to be attributed to the different statistical methods employed for detecting DEGs. Swanson *et al* (8) screened the DEGs using Partek software and the analysis of variance method, which calculates a *q*-value as described by Storey and Tibshirani (23), while the present study used the Limma package in R language, which is at present most commonly applied for identifying DEGs. To avoid false-positive results, relatively high cutoff values of FDR<0.05 and |log (fold change)|≥1.5 were set, which ensured high accuracy of the results.

The upregulated genes in the VAP group identified in the present study were mainly enriched in the GO terms of immune system processes and immune responses, which have been verified to have a pivotal role in the development of VAP (24). The immune system has a central role in controlling the duration and amplitude of the inflammatory response (25). A previous study on sepsis revealed that the innate and adaptive immune response is considerably different between

Table IV. GO annotation for genes in the top two modules.

Category	GO ID	Function	Count	P-value
Module 1				
BP	GO:0050832	Defense response to fungus	3	3.87×10^{-7}
	GO:0009620	Response to fungus	3	1.42×10^{-6}
	GO:0009617	Response to bacterium	5	1.96×10^{-6}
	GO:0051704	Multi-organism process	7	5.46×10^{-6}
	GO:0051707	Response to other organisms	5	2.53×10^{-5}
CC	GO:0005576	Extracellular region	9	8.31×10^{-8}
	GO:0031012	Extracellular matrix	3	0.0016
	GO:0044421	Extracellular region part	4	0.0031
	GO:0030141	Secretory granule	2	0.0103
	GO:0005615	Extracellular space	3	0.0114
MF	GO:0004252	Serine-type endopeptidase activity	3	5.05×10^{-5}
	GO:0008236	Serine-type peptidase activity	3	7.44×10^{-5}
	GO:0017171	Serine hydrolase activity	3	7.70×10^{-5}
	GO:0004175	Endopeptidase activity	3	0.0007
	GO:0004867	Serine-type endopeptidase inhibitor activity	2	0.00103
Module 2				
BP	GO:0071478	Cellular response to radiation	4	4.48×10^{-7}
	GO:2000379	Positive regulation of reactive oxygen species metabolic process	3	9.14×10^{-7}
	GO:0048519	Negative regulation of biological processes	9	1.27×10^{-6}
	GO:0009605	Response to external stimuli	7	1.63×10^{-6}
	GO:0071479	Cellular response to ionizing radiation	3	2.02×10^{-6}
CC	GO:0005634	Nucleus	7	0.0038
	GO:0005654	Nucleoplasm	3	0.0219
MF	GO:0004708	Mitogen-activated protein kinase kinase activity	2	3.65×10^{-5}
	GO:0004712	Protein serine/threonine/tyrosine kinase activity	2	0.00018
	GO:0004672	Protein kinase activity	3	0.0038
	GO:0016773	Phosphotransferase activity, alcohol group as acceptor	3	0.0064
	GO:0016301	Kinase activity	3	0.0079

GO, gene ontology; BP, biological process; CC, cellular components; MF, molecular function.

Table V. KEGG pathway enrichment for genes in Module 2.

KEGG ID	Function	Count	P-value
4380	Osteoclast differentiation	3	0.0005
5014	Amyotrophic lateral sclerosis	2	0.0022
4115	P53 signaling pathway	2	0.0035
5140	Leishmaniasis	2	0.0040
4370	Vascular endothelial growth factor signaling pathway	2	0.0044
4010	Mitogen-activated protein kinase signaling pathway	3	0.0044
4664	Fc epsilon RI signaling pathway	2	0.0048
4912	Gonadotropin-releasing hormone signaling pathway	2	0.0077
4620	Toll-like receptor signaling pathway	2	0.0078
4110	Cell cycle	2	0.0114
5145	Toxoplasmosis	2	0.0129
5160	Hepatitis C	2	0.0132

KEGG, Kyoto Encyclopedia of Genes and Genomes.

patients with VAP and those without this type of nosocomial infection (26). A cohort study including 90 patients with VAP mainly caused by Gram-negative bacteria demonstrated an association between immune system disorders and mortality, as patients with early monocyte apoptosis of >50% were less likely to succumb to sepsis compared with those exhibiting monocyte apoptosis of l<50% (27). The present study revealed that GO:0002376 (immune system processes) and GO:0006955 (immune responses) were significantly abnormal in patients with VAP, and the two GO categories included the genes *ELANE* and *LTF*. *ELANE* is the gene encoding neutrophil elastase, a serine protease expressed in myelomonocytic cells and their precursors (28). Mutations in the *ELANE* gene are proven to be the most common genetic cause of congenital neutropenia, as *ELANE* mutations are associated with functional deficiencies in neutrophils and further contribute to the risk of infection (29,30). In addition, lactoferrin, encoded by *LTF*, was suggested to affect the regulation of the inflammatory response by modulating cellular iron homeostasis (31). Furthermore, the present study showed that *ELANE* and *LTF* were the significant nodes in the resulting PPI network. It is therefore inferred that *ELANE* and *LTF* may be involved in the susceptibility of patients to VAP by affecting the immune response.

The present study also observed that the upregulated genes in patients with VAP were significantly enriched in the MAPK signaling pathway, which was also the most significant pathway enriched among genes in Module 2 mined from the PPI network. The MAPK signaling pathway is a three-tiered cascade that includes a MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K) and MAPK, which regulate numerous cellular functions, including proliferation, differentiation, migration and apoptosis (32). The four major MAPKs are extracellular regulated kinases 1 and 2 (ERK1/2), c-Jun-N-terminal kinases, p38 and ERK5 (33). Functional analysis performed in the present study demonstrated that four DEGs (*GADD45A*, *MAPK14*, *IL1R2* and *MAP2K6*) were significantly enriched in this pathway, among which *MAPK14* is also one of the nodes with the highest connection degree in the PPI network. *MAPK14* encodes the MAPK14 protein that is also referred to as p38α (34). p38α is the first identified member of the p38 MAPK family and can be targeted by pyridinylimidazole drugs that inhibit the production of pro-inflammatory cytokines (35). Höcker *et al* (36) have demonstrated that following its activation by the GADD45B-MAP3K4 signaling complex, MAPK14 is directed to autophagosomes, where it impairs autophagosome-lysosome fusion and thus autophagy. It has been indicated that activation of autophagy in macrophages mediates the early lung inflammation during mechanical ventilation via NLRP3 inflammasome signaling (37). These findings combined with the results of the present study lead to the hypothesis that the p38 MAPK signaling pathway may be involved in the development of VAP and *MAPK14* may be one of the key genes in this process.

In conclusion, the present study analyzed the gene expression profiles between patients with VAP and those not affected by this disease using a computational bioinformatics approach. Functional annotation of the DEGs into GO terms and KEGG pathways was performed, and a PPI network was constructed, followed by module mining. Genes including *ELANE*, *LTF* and *MAPK14* may have important roles in the development of VAP via altering the immune response and the MAPK signaling pathway. The present study provided a novel, genetic perspective on VAP, which may aid in the development of strategies to prevent or treat VAP in patients in ICUs.

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