Gene expression analysis for pneumonia caused by Gram-positive bacterial infection

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Abstract. Gram-positive bacteria are an important pathogenic factor for bacterial pneumonia. The aim of the present study was to identify the differentially expressed genes (DEGs) and to explore their associated pathways or expression patterns. Expression profiling of gene arrays from two independent datasets, GSE6269 and GSE35716, were downloaded from the Gene Expression Omnibus. The DEGs between peripheral blood samples from healthy controls and patients with bacterial pneumonia were identified. The Functional Annotation Tool in the Database for Annotation, Visualization and Integrated Discovery was used to annotate and analyze the DEGs in Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Multiple proteins were used to generate a protein-protein interaction (PPI) network. A total of 624 (621 annotated) were identified in the GSE6269 dataset and 398 (295 annotated) DEGs were identified in the GSE35716 dataset between pneumonia and healthy samples. A total of 40 common DEGs were identified between the 2 datasets, including 4 downregulated and 32 upregulated DEGs. In the GO category cellular component, melanosome was highly enriched among 11 genes; in the category biological process, the three most enriched items were regulation of ruffle assembly, negative regulation of calcium ion transport and necroptotic process. In the KEGG terms, only the nuclear factor-κB signaling pathway (*Homo sapiens* 04064) was significantly enriched. In the PPI network, five genes (CCL4, TIMP metallopeptidase inhibitor 1, intercellular adhesion molecule 1, plasminogen activator, urokinase receptor and cathepsin B) were identified to have a high degree of interaction with other DEGs. In conclusion, these five genes may represent key genes associated with pneumonia caused by Gram-positive bacteria. All of these results provide primary information and basic knowledge to understand the mechanisms of the pathogenesis.

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Introduction

Pneumonia refers to inflammation in the lower airways, the alveolar and pulmonary interstitium, which is caused by micro-organisms (bacteria, viruses or fungi) (1,2), physical and chemical factors, immune damage, allergies or drugs. Bacterial pneumonia is one of the most common forms of pneumonia, as well as one of the most common infectious diseases. Numerous types of bacteria may cause bacterial pneumonia, including *Streptococcus* (*S.*) *pneumoniae, Klebsiella pneumoniae, Haemophilus influenza* and *Pseudomonas aeruginosa* (3,4).

Gram-positive pathogens are particularly responsible for the increasing frequency of pneumonia (5). Bacteria are the most common cause of pneumonia in adults, while this pathology tends to be more severe in patients below the age of 5 and above the age of 65 years. Furthermore, patients with heart failure, diabetes, chronic obstructive pulmonary disease a or weak immune system due to human immunodeficiency virus infection/acquired immune deficiency syndrome or cancer chemotherapy also have a high risk of contracting bacterial pneumonia (6). At present, the majority of adult patients with bacterial pneumonia are successfully cured. In 1955, the mortality of this disease was as low as <10% (7) in patients of all ages. However, in infants and elderly people, bacterial pneumonia remains a lethal lung disease. The mortality rate increases from 1.3% (in patients <45 years) to 26.1% (in patients aged ≥85 years).

As the efficiency of the treatment of bacterial pneumonia is still dependent on the proper use of antibiotic drugs, an accurate diagnosis to distinguish between Gram-positive and the Gram-negative pathogens appears to be vital for the success of pneumonia treatment. Approaches including restriction of antibacterial drugs and appropriate medicinal therapy have important roles in improving the survival or cure rate of bacterial pneumonia. All of these rely on an accurate diagnosis and evaluation of the prognosis. Thus, it is vital to gain more insight into the pathogenesis of pneumonia, which may improve the identification of the pathogen and evaluation of the stage of this disease.

In addition, the incidence of bacterial pneumonia has markedly increased and the prognosis remains poor due to the

increasing resistance of several bacterial strains to antimicrobial agents (8-11). The Tracking Resistance in the United States Today (TRUST) study revealed that >18% of *S. pneumoniae* isolates were penicillin-resistant in 2001 (12). Therefore, the rapid development of novel types of medicine for bacterial pneumonia is in demand.

With the increasing elucidation of the mechanisms of the recognition and clearance of bacteria by the immune system, it has become apparent that pneumonia may alter certain dysregulated genes and bio-functional pathways in the lungs or in organs next to the site of the primary infection. A study on Gram-negative pneumonia identified an increased expression Toll-like receptor 2 and 4, as well as MD2, the determination of which contributed to the accurate diagnosis of pneumonia patients with sepsis (13). Analytic methods and strategies for generating gene expression profiles represent popular and feasible means of biomarker exploration in numerous cancer types. However, few studies were performed to screen the differentially expressed genes (DEGs) in bacterial pneumonia and the bio-functional pathways they participated in.

In the present study, these strategies were applied to Gram-positive pneumonia by using two independent gene expression datasets. The DEGs in peripheral blood mononuclear cells between pneumonia patients and healthy samples were identified. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to annotate and analyze the DEGs and identify those enriched in the Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Finally, a protein-protein interaction (PPI) network was mapped to identify key genes and/or pathways. All of these results provided primary information and basic knowledge to understand the mechanism of the pathogenesis. Furthermore, elucidation of the pathogenesis of bacterial pneumonia may contribute to the development of novel treatments.

Materials and methods

Data sources. Expression profiles of gene arrays were downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). Two independent datasets, GSE6269 (14) and GSE35716 (15), were selected to analyze the DEGs. The dataset GSE6269 consisted of 44 samples from the blood leukocytes of pediatric patients with *Streptococcus pneumonia* infection and 7 unrelated healthy controls based on the platform Affymetrix Human Genome U133 Array (Affymetrix; Thermo Fisher Scientific, Inc, Waltham, MA, USA). A total of 10 pneumonia samples from peripheral blood mononuclear cells stimulated with plasma from patients with bacterial pneumonia (Gram-positive) *in vitro* and 18 healthy control samples from the dataset GSE35716 were processed on the platform Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix; Thermo Fisher Scientific, Inc.).

Quality assessment. Quality assessment of the gene array datasets was performed with the affyPLM package (16) by using the linear modeling procedures at probe level. AffyRNAdeg was then used for the degradation of RNA. Relative log expression (RLE) and normalized unscaled standard errors were determined to assess the consistency of the data trends. Finally, only the data with a consistent trend as well as high RNA quality were included in the analysis.

Data preprocessing. In order to maintain the integrity and comparability of the data, the gcrma package (17) was used for normalization and adjustment to eliminate system errors in and between chips. As an important indicator to evaluate the reliability of experiments and sample selection, the correlation between gene expression levels was analyzed. Based on the Pearson correlation coefficient, a correlation chart for all the samples within one dataset was obtained.

Screening of DEGs. DEGs between patient and healthy samples were identified by using the Limma package (18). Gene expression was presented as logarithmic values. The threshold was log2 (fold change) >1 and $P<0.05$. Subsequently, the differences were visualized in a volcano plot, Venn diagram and Heat map by using ggplot2 (19), Venn diagram (20) and pheatmap (21) in R language.

Functional analysis of DEGs. Functional annotation tools in DAVID were used to annotate and analyze the associated pathways and functions of the DEGs (22). Furthermore, GO terms and KEGG pathways in which the key genes were enriched were determined. P-values were adjusted by using the Benjamini method (23) or the false discovery rate in multiple testing calibrations. The threshold was $P<0.05$.

PPI analysis. STRING (http://string-db.org/), the functional protein association networks, was used to construct and analyze the interactions between the proteins encoded by these DEGs. Hereinto, multiple proteins were applied to map the PPI network (24).

Results

Data source and quality assessment. Regression analysis of the raw data from the two databases was performed to control the data quality. Corresponding boxplots of the RLE were generated to verify the homogeneity between chips by using affyPLM in R. The majority of the data-points representing samples from the GSE6269 and GSE35716 datasets centered around 0, having approximately the same dispersion. The quality of each dataset was appropriate for the subsequent analysis.

Data preprocessing. The gcrma package was then applied to normalize the original data of the samples from the two datasets. Based on the density histograms and boxplots of log-intensities of normalized data from GSE35716 and GSE6269, the relative expression of samples from the two databases ranged from 0 to 15, revealing a reasonably small extent. The general expression of the DEGs in the GSE6269 and GSE35716 dataset concentrated at around 2, indicating a similar expression trend.

After normalization, logarithmic expression values were subjected to Pearson correlation analysis of samples with cor functions in R. The graphs of the correlation clustering (between different genes in each sample) indicated that the expression of each sample in the GSE6269 as well as the GSE35716 dataset

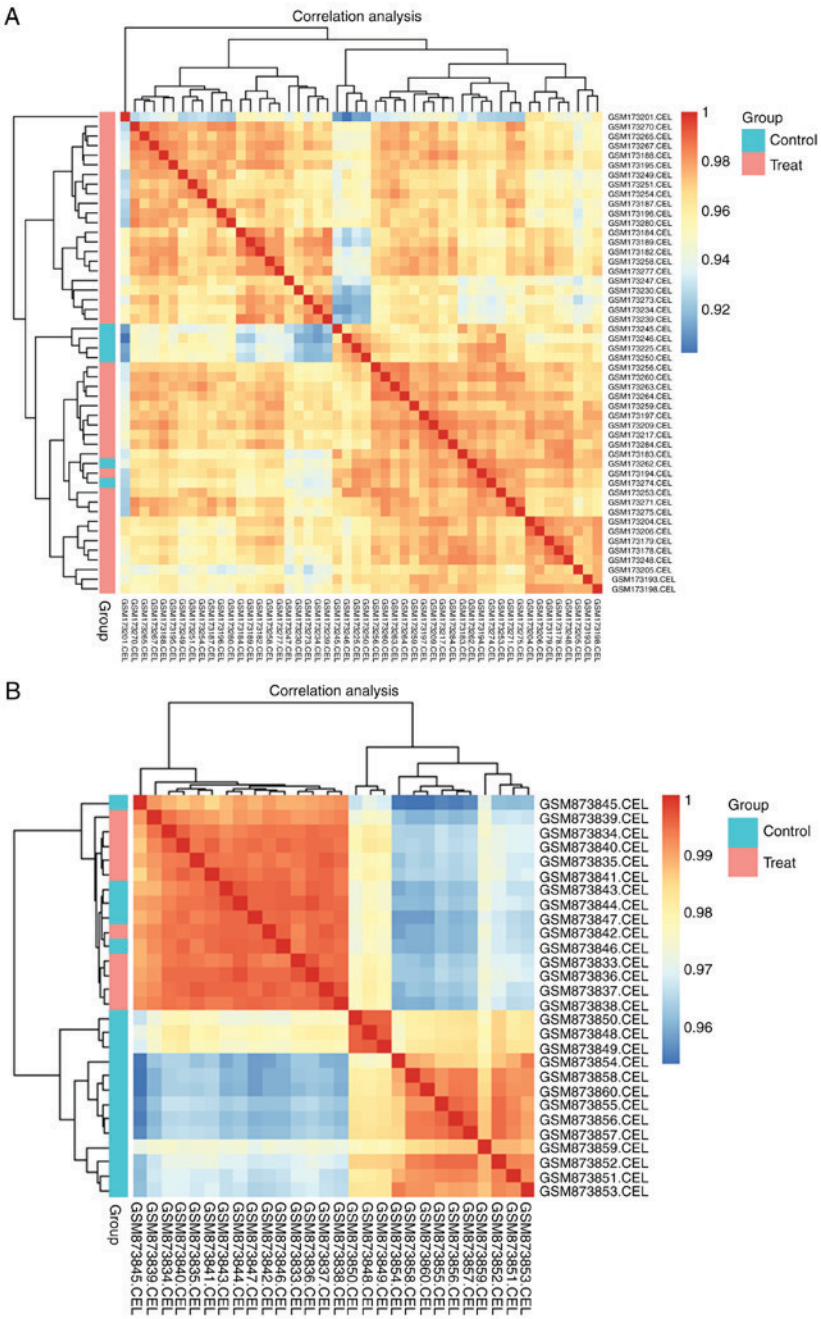

Figure 1. Correlation analysis of samples after normalization. (A) Clustering analysis based on the Pearson correlation coefficient of normalized sample data from GSE6269. Control group, blood leukocytes from healthy subjects; treatment group, blood leukocytes from patients with *Streptococcus pneumoniae* infection. (B) Clustering analysis based on the Pearson correlation coefficient of normalized sample data from GSE35716. The deeper red represents a higher correlation. Control group, peripheral blood mononuclear cells stimulated with plasma from healthy individuals; treatment group, peripheral blood mononuclear cells stimulated with plasma from patients with bacterial pneumonia.

was highly correlated (Fig. 1). The minimum correlation coefficients of samples in GSE6269 and GSE35716 were 0.901 and 0.953, respectively. However, samples in the control group and treat group could not be clustered significantly for each of the two databases. However, when using the first three principal components for principal component analysis (Fig. 2), the clustering of samples in the two databases was consistent with the correlation analysis.

DEGs. In the dataset GSE6269, a total of 624 DEGs were identified between pneumonia samples and healthy samples, including 323 upregulated and 301 downregulated genes. Hereinto, 621 genes were annotated (Fig. 3A). By comparing 10 pneumonia samples with 18 healthy samples from the dataset GSE35716, 398 DEGs and annotation information for 295 of them were obtained (Fig. 3B). These DEGS were comprised of 289 significantly upregulated and 109 downregulated genes. Among these the two different databases, 40 common genes were identified. Except for the four genes [adenosine deaminase, C-C motif chemokine ligand 4 (CCL4), chloride intracellular channel 3 and inhibitor of DNA binding 3] that exhibited different expression patterns in the two datasets, a total of 4 common downregulated and 32 common upregulated DEGs were identified (Table I).

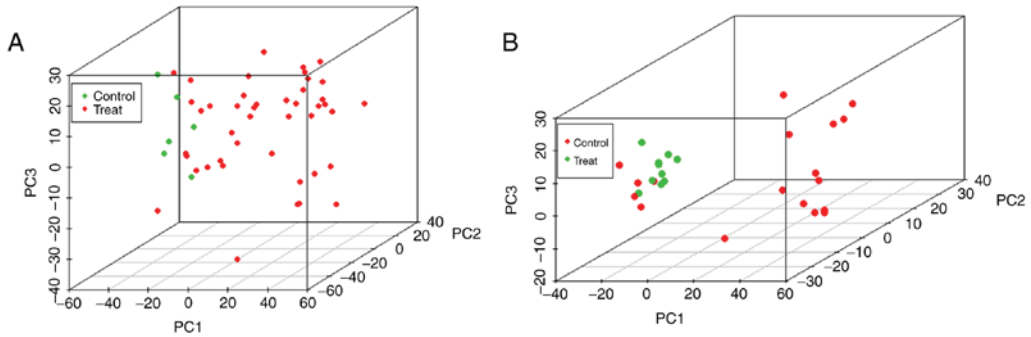

Figure 2. Distribution of samples according to aPC analysis. (A) Distribution of samples in GSE6269. Control group, blood leukocytes from healthy subjects; treatment group, blood leukocytes from patients with *Streptococcus pneumoniae* infection. (B) Distribution of samples in GSE35716. Control group, peripheral blood mononuclear cells stimulated with plasma from healthy individuals; treatment group, peripheral blood mononuclear cells stimulated with plasma from patients with bacterial pneumonia. The red dots represent samples from the treat group and the green dots represent samples from the control group. The PC1 is displayed on the x-axis, the PC2 is displayed on the z-axis and the PC3 is displayed on the y-axis. PC, principal component.

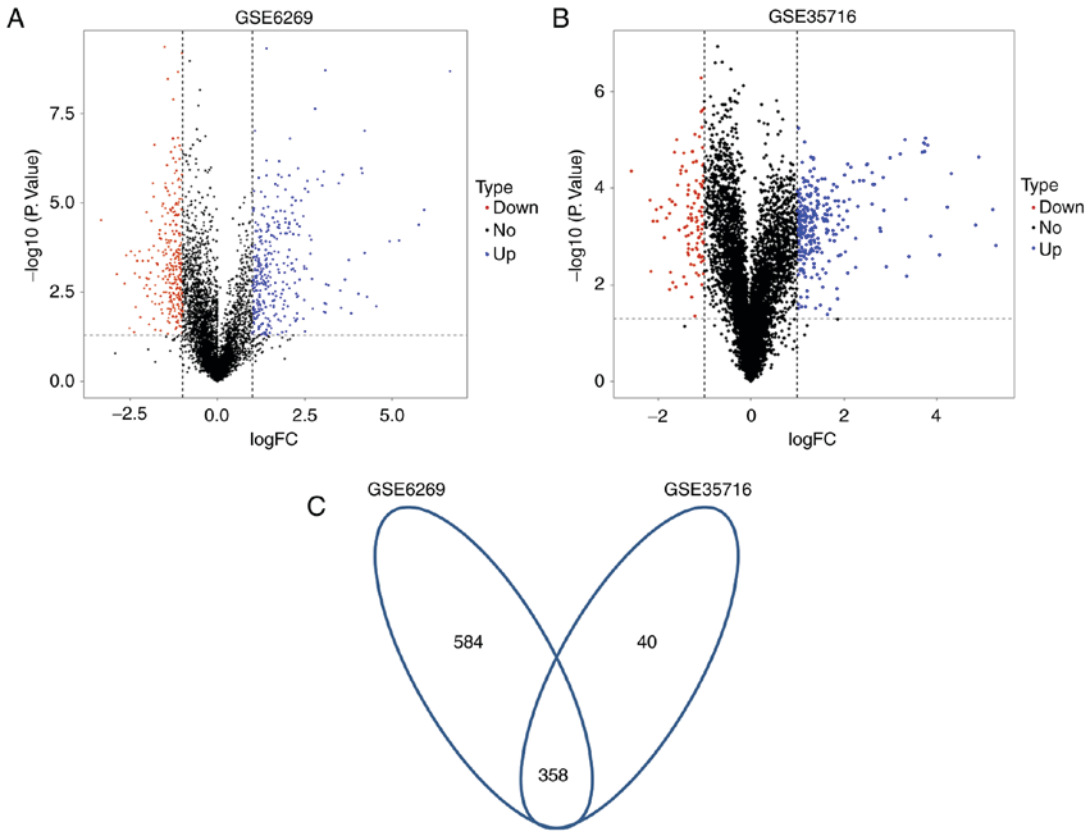

Figure 3. Identification of DEGs in samples from the GSE6269 and GSE35716 datasets. Volcano plots for the identification of DEGs in (A) the GSE6269 and (B) the GSE35716 dataset. Each data-point represents a gene from the individual gene expression profile. Data-points highlighted in blue represent genes which were upregulated and the ones displayed in red were downregulated. The black data-points marked as 'no' are the genes without any significant difference in expression. The log10 ratio of expression (normal/pneumonia) is displayed on the y-axis and the logFC is displayed on the x-axis. (C) Common DEGs from the two platforms. DEG, differentially expressed gene; FC, fold change.

Functional enrichment analysis. Functional enrichment analysis of 40 common DEGs was performed using DAVID (Fig. 4). GO analysis revealed that the DEGs were significantly enriched in 32 GO terms, including 16 terms in the category biological process (BP), 11 terms in the category cellular component (CC) and 4 terms in the category molecular function. In the CC category, melanosome was highly enriched among the 11 terms and in the category BP, the three most enriched items were regulation of ruffle assembly, negative regulation of calcium ion transport and necroptotic process. No DEGs were enriched in the molecular function (MF) catagory. Among the KEGG terms, only the nuclear factor (NF)-κB signaling pathway (*Homo sapiens* 04064) was significantly enriched.

PPI analysis. To explore the biological and regulating functions of the common DEGs at the protein level, a PPI network was constructed identify the key genes associating

Table I. Common genes from the two platforms.

Regulation	Differentially expressed genes
Upregulated	PLAUR, ADAP2, TREM1, TGFBI, CAPG, PPIF, NPL, EREG, LGALS1, TIMP1, PILRA, CTSB, THBD, PKM, SIRPA, TNFAIP2, ICAM1, FCAR, LILRB1, CD14, S100A12, MAPKAPK3, PLEC, CES1, IER3, BSG, ANPEP, CORO1C, TOM1, MGLL, GRN, APLP2
Downregulated	ETS1, IPCEF1, ABHD10, GVINP1

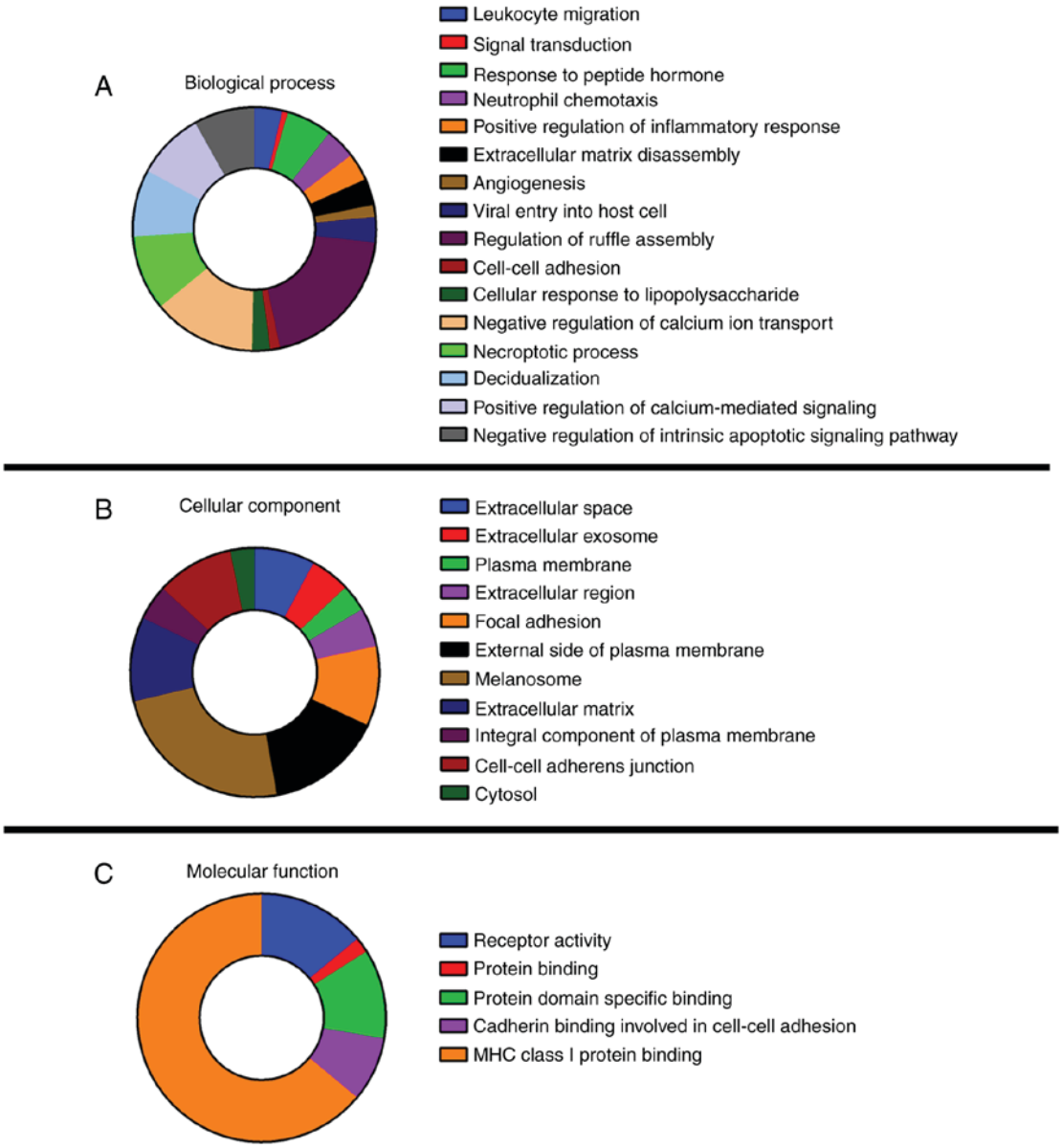

Figure 4. GO functional enrichment analysis of the 40 common differentially expressed genes. Significantly enriched terms in the GO categories. (A) Biological process; (B) cellular component; and (C) molecular function. The relative area represents the degree of enrichment. GO, Gene Oncology; MHC, major histocompatibility complex.

with pneumonia caused by Gram-positive bacteria (Fig. 5). Analysis with STRING identified 19 genes that interacted with each other, generating 19 PPIs. This PPI network was visualized by using Cytoscape, and hub genes with a degree of interaction of >3 were selected for further analysis. Five genes [CCL4, TIMP metallopeptidase inhibitor 1 (TIMP1), intercellular adhesion molecule 1 (ICAM1), plasminogen activator, urokinase receptor (PLAUR) and cathepsin B (CTSB)] were identified as hub genes that strongly interacted with other DEGs (Table II). These five genes may represent key genes associated with pneumonia caused by Gram-positive bacteria.

Table II. Annotation information of the five key differentially expressed genes.

Gene symbol	GO terms
CCL4	Extracellular space, signal transduct binding, neutrophil chemotaxis, positive regulation of inflammatory response, positive regulation of calcium-mediated signaling
TIMP1	Extracellular space, extracellular region, protein binding, extracellular exosome, response to peptide hormone, extracellular matrix disassembly
ICAM1	Extracellular space, protein binding, extracellular exosome, leukocyte migration, receptor activity, plasma membrane, focal adhesion, external side of plasma membrane, viral entry into host cell, regulation of ruffle assembly, integral component of plasma membrane
PLAUR	Protein binding, extracellular exosome, receptor activity, plasma membrane, focal adhesion, integral component of plasma membrane, signal transduction, protein domain specific binding, negative regulation of intrinsic apoptotic signaling pathway
CTSB	Extracellular space, protein binding, extracellular exosome, viral entry into host cell, extracellular region, melanosome, decidualization, receptor activity

GO, Gene Ontology; CCL4, C-C motif chemokine ligand 4; TIMP1, TIMP metallopeptidase inhibitor 1; ICAM1, intercellular adhesion molecule 1; PLAUR, plasminogen activator, urokinase receptor; CTSB, cathepsin.

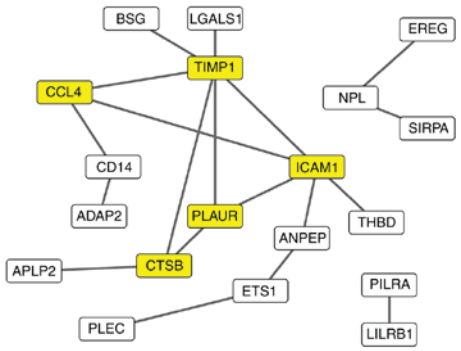

Figure 5. PPI network of common differentially expressed genes. The boxes represent the gene nodes, the connecting lines represent the interactions and boxes in yellow indicate hub genes with a degree of interaction of >3. PPI, protein-protein interaction; CCL4, C-C motif chemokine ligand 4; TIMP1, TIMP metallopeptidase inhibitor 1; ICAM1, intercellular adhesion molecule 1; PLAUR, plasminogen activator, urokinase receptor; CTSB, cathepsin B.

Discussion

According to the statistics from the World Health Organization, pneumonia causes ~1.6 million deaths annually, becoming a leading cause of morbidity and mortality throughout the world (25,26). Among the casualties of pneumonia, >1 million are children under the age of 5 years. An estimate of 90% of pneumonia-associated deaths occur in developing countries. Individuals aged >60 years are also a major population affected by pneumonia. Gram-positive bacteria are accountable for a large proportion of all severe pneumonia cases, including nosocomial pneumonia and community-acquired pneumonia (27).

Bacteria are commonly present in parts of the upper respiratory tract; however, they are able to enter alveolar spaces between the cells and also travel between adjacent alveoli through connecting pores (28). This invasion triggers an immune response, comprising the recruitment of white blood cells (neutrophils) with the capacity to attack microorganisms to the lungs. A general activation of the immune system is then triggered by the neutrophils and cytokines. The neutrophils, bacteria and fluid leaked from surrounding blood vessels fill the alveoli and result in impaired oxygen transportation (29). However, further details regarding the mechanism of the immune response to Gram-positive bacterial pneumonia remain to be elucidated.

At present, the diagnostic efficacy of Gram-positive pneumonia is far from satisfactory, and drug resistance among Gram-positive organisms is now a serious therapeutic problem despite the availability of novel antimicrobials. An improved knowledge of the immune mechanisms associated with pneumonia caused by Gram-positive organisms may contribute to the effective treatment and the development of more immunogenic vaccines.

At present, advanced biological techniques, including gene array and high-throughput sequencing are ideal approaches to assess the mechanisms of the development and immune responses to various diseases. In the present study, a bioinformatics analysis of gene array datasets was applied to determine DEGs in Gram-positive pneumonia and their associated pathways. Two independent datasets, GSE6269 and GSE35716, were selected, which contained gene expression profiles of peripheral blood samples from healthy controls and patients with bacterial pneumonia. A total of 40 common DEGs associated with pneumonia were identified between the two databases. All of these DEGs were annotated subjected to GO/KEGG functional enrichment analysis by using DAVID. Key DEGs, including CCL4, TIMP1, ICAM1, PLAUR and CTSB, were further mapped in a PPI network.

CCL4/macrophage inflammatory protein-1β (MIP-1β) is a CC chemokine with specificity for C-C chemokine receptor type 5 receptors. As a chemoattractant for a variety of other immune cells, including natural killer cells and monocytes, CCL4/MIP-1β has a vital role in inflammation caused by bacteria and viruses (30). It was also identified to be induced by Gram-positive bacteria including *Lactococcus lactis* (31). As an addition to a previous study reporting that CCL4 interacted

with CCL3 (32), the present results also indicated that CCL4 interacted with ICAM1 and TIMP1 in Gram-positive pneumonia. ICAM-1/CD54, a protein encoded by the ICAM1 gene in humans (33,34), is a cell surface glycoprotein typically expressed on endothelial cells and cells of the immune system, binding to integrins of the type CD11a/CD18 or CD11b/CD18. Thus, it is associated with a series of immune responses in inflammatory diseases. Studies have identified that ICAM-1 was significantly differentially expressed in *S. pneumoniae* infection and bacterial or viral meningitis (35,36). Another gene interacting with CCL4 is TIMP1, a tissue inhibitor that regulates matrix metalloproteinases and disintegrin-metalloproteinases [a disintegrin and metalloproteinase (ADAMs) and ADAMs with thrombospondin motifs] (37). Studies have reported that TIMP1 was dysregulated in numerous types of lung cancer (38,39), breast cancer (40) and nephritis (41,42). In accordance with the results of the present study, TIMP1 was also identified to be associated with interstitial pneumonia (43,44).

CTSB belongs to a family of lysosomal cysteine proteases and is encoded by the CTSB gene in humans (45,46). It is an important endogenous protease in intracellular proteolysis, regulating cell apoptosis and restricting injury-associated inflammation (47). This protein was identified to be upregulated in premalignant lesions and various pathological conditions, as well as in cancer. PLAUR, also known as urokinase-type plasminogen activator (uPA) receptor or uPAR/CD87, is a multi-domain glycoprotein tethered to the cell membrane. It was reported to have important roles in processes associated with numerous diseases, including tumor infiltration (48) and inflammation (49).

In conclusion, the present study identified five key DEGs in Gram-positive pneumonia. The type of bioinformatics analysis performed in the present study has been rarely applied to study this disease. The results indicated that these five key genes had a high degree of interaction. It may be suggested that ICAM1, TIMP1 and CCL4 co-function in Gram-positive bacterial pneumonia by participating in the regulation of the NF-κB signaling pathway. However, further laboratory experiments are still required to confirm the exact association between two correlating genes to clearly understand what correlation patterns existing between them. The present study provided basic information paving the road for future experimental research to explore the mechanisms of the development of Gram-positive bacterial pneumonia. The increasing knowledge regarding the mechanisms of this disease may lead to the improvement of the diagnostic efficacy, as well as the development of novel treatments.

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