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Bronchoalveolar lavage proteomics in exacerbation of bronchiectasis

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Abstract

Background The molecular pathophysiology underlying the development of bronchiectasis with exacerbation at the proteomic level has not been clarified using bronchoalveolar lavage fluid samples. This study aimed to evaluate the bronchoalveolar lavage fluid inflammatory profiles associated with exacerbation of bronchiectasis.

Methods We analyzed the bronchoalveolar lavage fluid specimens from 4 patients in the exacerbation status and 4 patients in a stable status using liquid chromatography-tandem mass spectrometry.

Results A total of 1,577 proteins were identified using proteomic analysis, with 127 differentially expressed proteins. Of 127 differentially expressed proteins, 23 proteins showed more than 2-fold differences between exacerbation and stable status groups. The exacerbation status was associated with 18 upregulated proteins (TPI1, CRP, BPI, ORM1, PTPRE, S100A9, BPY2, TPM4, ERVFC1-1, CYS1, CLEC3B, S100A8, PSAT1, NDUFA10, MDGA1, SPRR3, ALDOA, and PSMB2) and five downregulated proteins (MUC5B, HSPE1, KLK13, IGHA1, and MUC5AC). Pathway analysis revealed that the neutrophil degranulation pathway (R-HSA-6798695) was the most enriched pathway in these proteins, followed by the C-type lectin receptor pathway (R-HSA-5621481).

Conclusion The bronchoalveolar lavage fluid protein expression in patients in the exacerbation status of bronchiectasis was significantly different from that in patients in the stable status, indicating that neutrophil degranulation and C-type lectin receptor pathways are the most enriched pathways during exacerbation.

Keywords Bronchiectasis, Bronchoalveolar lavage, Proteomics, Neutrophil degranulation

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Introduction

Bronchiectasis is a chronic respiratory disorder characterized by irreversible dilation and damage to the bronchial walls, leading to impaired mucus clearance and increased susceptibility to recurrent bronchial infections [1]. The prevalence and socioeconomic burden of bronchiectasis have increased worldwide [2–4]. The exacerbation (AE) of bronchiectasis, defined as a sustained worsening of the respiratory symptoms, is associated with poor treatment outcomes, and the prevention and/or appropriate management of AE is crucial for altering the natural course of this disease [5, 6]. Exacerbations are now increasingly understood through the lens of the "vicious vortex" model, which describes a cycle of infection, inflammation, impaired mucociliary clearance, and structural damage. Accordingly, understanding the mechanisms underlying AE development is imperative for the development of novel treatments to reduce the occurrence of AE in bronchiectasis.

Proteomics is a powerful and rapidly evolving field of study that offers an innovative lens for examining the intricate molecular signatures and protein dynamics [7]. Unlike genomics, which provides information about the genetic blueprint of an individual, proteomics enables the direct analysis of protein expression, modifications, and interactions within a biological system [8, 9]. Thus, proteomic investigations can yield invaluable insights into the functional alterations in cellular pathways, which are crucial to understanding the pathogenesis of bronchiectasis.

Previous studies evaluating the role of proteomics in the development of AE in bronchiectasis were performed using sputum samples [10–12]. Although these studies have revealed important novel findings, sputum samples have limitations in terms of contamination and these may not reflect true local inflammation (in the active parenchymal area of bronchiectasis) [11]. Therefore, to overcome these limitations, we applied liquid chromatography-tandem mass spectrometry (LC-MS/MS), a high-throughput proteomic screening method, to bronchoalveolar lavage (BAL) fluid samples obtained from patients with bronchiectasis. Our study aimed to determine whether a high-throughput proteomic screening method could reveal differentially expressed proteins associated with the AE of bronchiectasis.

Materials and methods

Study population

This prospective study evaluated 8 patients with bronchiectasis between January 2021 and January 2022 at Chungbuk National University Hospital, a tertiary referral hospital in Cheongju, Republic of Korea. Bronchiectasis was diagnosed by two pulmonologists (BHY and SHK) based on respiratory symptoms and computed tomography findings, according to published guidelines [13, 14].

During the study period, 4 patients in a stable status and 4 patients in an AE status were enrolled in the study. No attempt to match for age, sex, or body mass index (BMI) was made, as prior studies on both chronic obstructive pulmonary disease (COPD) and bronchiectasis found no relationship between the sputum proteome and these parameters [10, 15]. The AE of bronchiectasis is defined as the worsening of three or more major symptoms for 48 h or more, necessitating a change in treatment [16]. Major symptoms include coughing, sputum volume/viscosity, sputum suppuration, dyspnea, exercise ability, fatigue, malaise, and hemoptysis. Moreover, we define a stable status as a condition in which a patient does not require treatment changes, including antibiotic or corticosteroid use, for 4 weeks or longer [16, 17]. The study protocol was approved by the Institutional Review Board of the Chungbuk National University Hospital (application no. 2021-10-007) in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrollment.

Primary outcomes

The primary outcome of this study was to identify differences in BAL proteomics and related pathways associated with the AE status of bronchiectasis.

Measurements of the clinical variables

The baseline demographic characteristics included the age, sex, BMI (weight in kilograms divided by the height in meters squared), and smoking status (never, former, or current smokers). Dyspnea was evaluated using the modified Medical Research Council scale [18]. The presence of patient-reported physician-diagnosed comorbidities was assessed at baseline. The white blood cell count, neutrophil count, and high-sensitivity C-reactive protein levels were measured in all patients.

Pre- and post-bronchodilator spirometry were performed according to the recommendations of the American Thoracic Society/European Respiratory Society [19]. The absolute values of forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were recorded; additionally, the percentages of the predicted values for FEV_1 (FEV_1%pred) and FVC (FVC %pred) were calculated using an automatic calculator by using a reference equation obtained from a representative Korean sample [20].

Microbiological analyses of specimens were performed using standard methods [21]. Conventional semi-quantitative bacterial and fungal cultures were also performed. All samples underwent initial Gram staining before culturing the BAL fluid if they met the Murray and Washington criteria [22].

Bronchoalveolar lavage

BAL fluid and microbiological analyses were conducted for all patients. BAL procedures were performed by two pulmonologists (BHY and SHK) according to the American Thoracic Society recommendations [23]. Bronchoscopy was performed using a standard flexible bronchoscope (BF-1TQ260; Olympus, Tokyo, Japan). During the BAL, the scope was placed in a wedge position within the selected segmental bronchi. A total volume of normal saline between 100 and 300 ml, divided into three to five aliquots, was instilled through the channel of the bronchoscope. After the instillation of each aliquot, the instilled saline was gently retrieved using a negative suction pressure of less than 100 mmHg to avoid visible airway collapse.

Comprehensive proteomic analysis of the Bronchoalveolar lavage samples of patients with bronchiectasis

Protein analysis of the BAL samples was conducted as illustrated in Fig. 1. The detailed process is described in the next sections.

Materials

The High-Select™ Top14 abundant protein depletion resin (catalog number A36370), which was used for removing the 14 abundant proteins from the BAL samples, and the Tandem Mass Tag (TMT) Pro 16-plex isobaric label reagent set were purchased from Thermo Fisher Scientific (Rockford, USA). An Amicon Ultra centrifugal filter (3 kDa MWCO) and S-Trap mini spin columns were obtained from Merck Millipore (Billerica, USA) and ProFit (New York, USA), respectively. Sodium dodecyl sulfate, 1,4-dithiothreithol (DTT), iodoacetamide (IAA), ammonium bicarbonate (ABC), ammonium acetate, and triethylammonium bicarbonate (TEAB) buffers were purchased from Sigma-Aldrich. Trypsin Gold (mass spectrometry grade) from Promega (Madison, WI, USA) was used.

Immunoaffinity depletion

Fourteen abundant proteins in the BAL samples (albumin, IgA, IgD, IgE, IgG, IgG light chains, IgM, alpha-1-acid glycoprotein, alpha-1-antitrypsin, alpha-2-macroglobulin, apolipoprotein A1, fibrinogen, haptoglobin, and transferrin) were removed using the High-Select™ Top14 resin following the manufacturer's protocol. Briefly, the depletion spin columns were equilibrated to room temperature, and 10 µl of samples were directly added to the resin slurry in the column. The samples were mixed by inverting the column several times until the resin was completely homogenous. The mixture in the column was incubated under gentle end-over-end mixing for 10 min at room temperature. After incubation, the contents of the mini column were added to a 1.5 ml collection tube and centrifuged at 1,000 × *g* for 2 min. The 14 most abundant proteins were removed from the collected samples. The samples were filtered

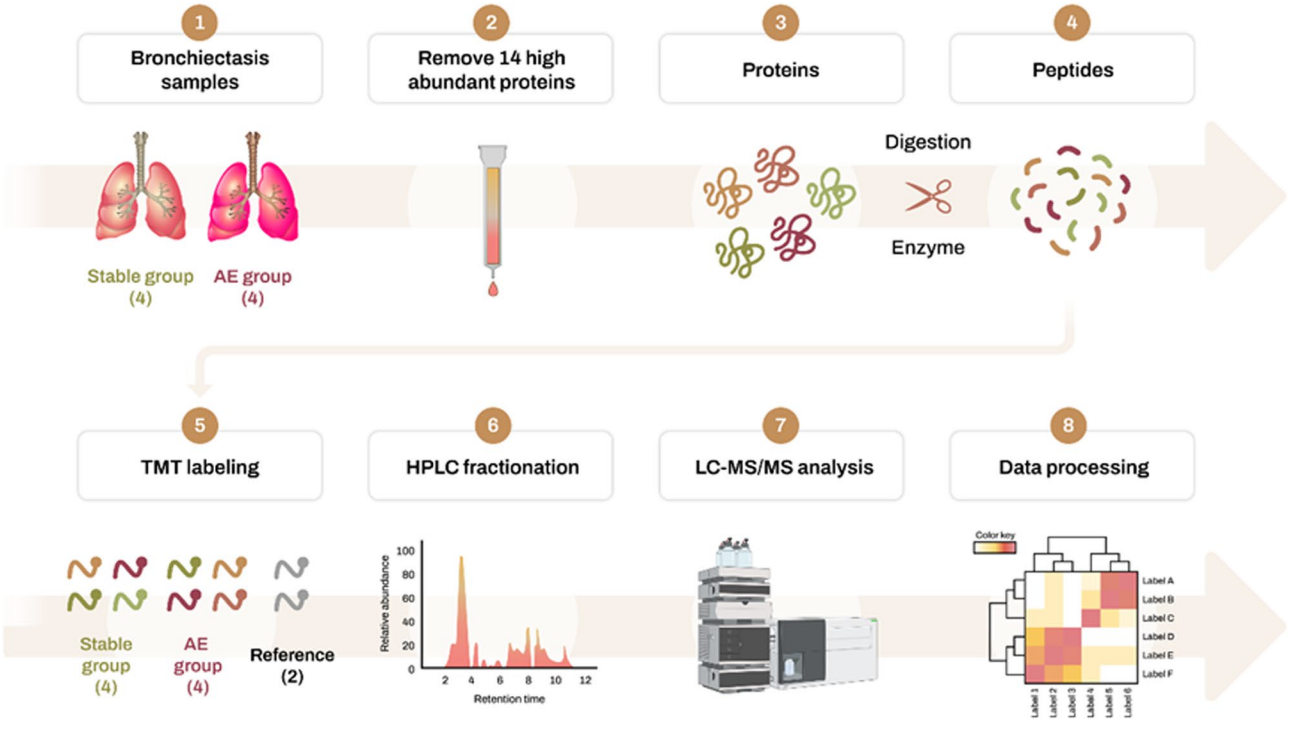

Fig. 1 Experimental workflow used for the proteome analysis of the BAL samples. Abbreviations: BAL, bronchoalveolar lavage

using an Amicon Ultra centrifugal filter (3 kDa MWCO) for concentration and desalting.

Proteome sample preparation

Depleted and desalted BAL samples were digested using S-Trap mini spin columns following the manufacturer's protocol. The proteins were denatured in sodium dodecyl sulfate and TEAB, reduced using TCEP, alkylated with IAA in the dark, and quenched with phosphoric acid. After adding the binding/wash buffer, the solution was centrifuged in the S-Trap column, washed, and digested with trypsin at 37 °C for 16 h. Peptides were eluted using three different buffers and centrifuged. The resulting peptide solutions were pooled, dried, and quantified using a BCA assay. These peptides were then labeled with TMT-16 plex reagents, and the reaction was quenched using a hydroxylamine solution. The labeled samples were combined and dried for further analyses.

High pH reversed-phase liquid chromatography for peptide fractionation

TMT-labeled samples were fractionated using an XBridge BEH Shield RP18 column on a Nexera XR high-performance liquid chromatography (HPLC) system, and 40 fractions were collected and concentrated into 10 fractions for LC-MS/MS analysis.

Liquid chromatography-tandem mass spectrometry

These ten fractions were analyzed using an Easy nLC 1200 system and an Orbitrap Fusion Lumos mass spectrometer equipped with a nanoelectrospray source. The samples were initially trapped in a C18 precolumn and then separated using a C18 analytical column at a flow rate of 250 nl/min. The mobile phase consisted of water (A) and an acetonitrile-water-formic acid mixture (B). The LC gradient varied from 5 to 95% for phase B over a specified time course. The mass spectrometer was operated in the data-dependent mode, alternating between MS and MS/MS scans. The MS spectra were collected at a resolution of 120,000, whereas the MS/MS spectra were collected at a resolution of 60,000 with specific settings for ion injection times, AGC target values, and collision energy. The system used a 30 s exclusion time for previously fragmented ions.

Pre-processing and data processing

Prior to the LC-MS/MS analysis, sample preparation involved protein trapping to remove the non-essential proteins and contaminants from the BAL samples, thus concentrating the necessary proteins for analysis. This process successfully eliminated 14 abundant plasma proteins, including albumin and immunoglobulins, with only the relevant proteins remaining. Subsequently, protein digestion was performed using trypsin. These peptides were labeled with isobaric TMT agents for detailed quantitative analysis. HPLC was used to fractionate the labeled samples into 10 parts, simplifying their complexity, before performing LC-MS/MS. This method enables the accurate measurement, quantification, and identification of peptides, thereby providing essential data on diagnostic markers and proteolytic processes.

Protein identification and quantitation

The Integrated Proteomics Pipeline with built-in search engines (IP2, version 6.5.5, Integrated Proteomics) was used for data analysis using the UniProt human protein database (September 2021, reviewed). Reverse sequences of all proteins were appended to the database to calculate the false discovery rate. ProLuCID [24] was used to identify the peptides with a precursor mass error of 5 ppm and a fragment ion mass error of 50 ppm. Trypsin was selected as the enzyme to use because of two potentially missed cleavages. The TMT modification (+304.2071) of the N-terminus and lysine residue using the labeling reagent and carbamidomethylation of cysteine were chosen as static modifications. Methionine oxidation was chosen as the variable modification. The reporter ions were extracted from small windows (approximately 20 ppm) around their expected m/z values in the HCD spectrum. The output data files were filtered and sorted to compose the protein list using DTASelect (The Scripps Research Institute, USA), with two or more peptide assignments for protein identification and a false positive rate of less than 0.01 [25].

Data processing

Quantitative analysis was conducted using Census in the IP2 pipeline (Integrated Proteomics, USA) with only unique peptides. The intensity of the reporter ion channel for a protein was calculated as the sum of the intensities of the reporter ions from all constituent peptides of the identified protein [26]. The reverse and potentially contaminating proteins were removed. The Perseus platform (version 2.0.5.0) was used for data processing. The intensity of the protein was input into Perseus, $\log_2$-transformed, and normalized to the median of the column for protein quantification. Student's t-test was performed to select the significant proteins with a p-value between the two groups below 0.05 to identify the significant proteins. Moreover, pathway analysis of the significantly differentially expressed proteins was performed to determine the functional proteins whose expression differed between the two groups. The Perseus software was used to perform unsupervised hierarchical clustering and principal component analysis (PCA), and to generate volcano plots (Fig. 2).

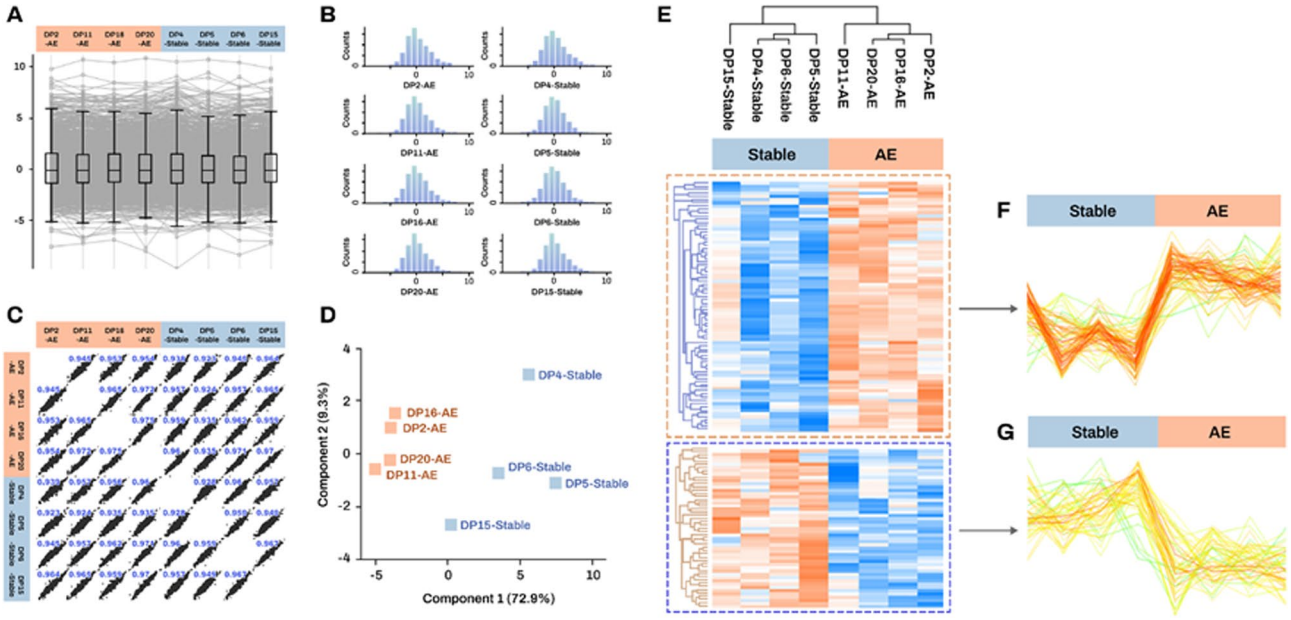

Fig. 2 The sequential steps of the statistical analysis performed. **a** The Tandem Mass Tag intensity obtained from the chromatography-tandem mass spectrometry analyses was log_2-transformed and normalized to the median of the column **b** The intensity versus frequency distributions for all eight samples are similar **c** The Pearson correlation coefficients among samples were over 0.9 **d** The principal component analysis could clearly discriminate the stable group from the AE group **e** Hierarchical clustering was performed for the 127 significant proteins between the two groups (p-value < 0.05) **f** and **g** show the upregulated and downregulated proteins in the AE group compared to stable group, respectively

Gene ontology (GO) pathway enrichment analysis

The proteins that remained altered following resuscitation in each tissue sample were subjected to GO pathway analysis using Metascape (http://metascape.org). Protein names were converted into Entrez gene IDs and redundant identifiers were merged into a single Entrez gene ID. Pathway and process enrichment analyses utilized various ontology sources, including GO biological processes, KEGG pathway, reactome gene sets, CORUM, PanGenBase, WikiPathways, and the PANTHER pathway. Significant terms ($p < 0.01$; minimum count of three; and an enrichment factor > 1.5) were clustered based on membership similarities and the most significant term within each cluster represented the respective cluster. P-values were calculated based on an accumulative hypergeometric distribution.

Statistical analysis of the clinical variables

Data are presented as the mean and standard deviation (SD) for the continuous variables and as frequency (percentage) for the categorical variables. Continuous variables were compared using the Mann–Whitney U test, and the Pearson chi-square test or Fisher's exact test was used to compare the categorical variables, as appropriate. All tests were two-sided, and p-values < 0.05 indicated statistical significance. All statistical analyses were performed using the IBM SPSS Statistics for Windows (version 27.0; IBM Corp., Armonk, NY, USA).

Results

Baseline characteristics

The baseline characteristics of the study population are summarized in Table 1. Among the 8 patients, 4 patients were in the AE group and four were in the stable group. No significant differences in the baseline characteristics were found between the AE and stable groups (all $P > 0.05$).

Proteome analysis

Normalization, correlation, PCA, and protein variation

In total, a total of 1,577 proteins were identified and quantified in stable and AE BAL samples using LC-MS/MS analysis, coupled with TMT labeling and HPLC fractionation into ten fractions (Supplemental Table 1). The intensity of the TMT reporter ions was extracted from the TMT labelled peptides with an accuracy of 25 ppm. Figure 2 shows the sequential steps of the statistical analysis. The protein intensity summed by the TMT reporter ions from the peptide was normalized to the median of each sample after the log_2 transformation (Fig. 2a). The intensity versus frequency histograms for all samples showed a Gaussian distribution (Fig. 2b). The Pearson correlation values among all four stable and four AE samples were over 0.9 (Fig. 2c).

PCA was performed on all 127 significantly differentially expressed proteins after performing Student's t-test between the two groups (Fig. 2d). It clearly discriminated two groups, with component 1 accounting for 72.9% of

Table 1 Baseline characteristics

	Total (*n* = 8)	AE status (*n* = 4)	Stable status (*n* = 4)	*P*-value
Age, years	61.62 (10.61)	58.00 (13.09)	65.25 (7.50)	0.374†
Sex, male	2 (25.0)	2 (50.0)	0 (0.0)	0.414‡
BMI (kg/m^2)	20.03 (4.08)	21.26 (5.71)	18.79 (1.46)	0.434†
Smoking history				
Current or ex-smoker	0	0	0	-
Previous history of TB	0	0	0	-
Comorbidities				
COPD	0 (0.0)	0(0.0)	0 (0.0)	-
Asthma	2 (25.0)	1 (25.0)	1 (25.0)	1.000‡
Cardiovascular disease	0	0	0	-
Diabetes mellitus	1 (12.5)	1 (25.0)	0 (0.0)	1.000‡
Chronic kidney disease	0	0	0	-
Neurologic disease	0	0	0	-
Autoimmune disease	1 (12.5)	1 (25.0)	0 (0.0)	0.876‡
Malignancy	1 (12.5)	0 (0.0)	1 (25.0)	1.000‡
NTM-PD	3 (37.5)	1 (25.0)	2 (50.0)	1.000‡
Spirometry				
FVC, L	2.03 (0.66)	2.03 (1.03)	2.03 (0.15)	0.996†
FVC, % predicted	66.02 (19.26)	60.42 (28.42)	71.62 (5.09)	0.539†
FEV1, L	1.58 (0.59)	1.50 (0.91)	1.65 (0.13)	0.792†
FEV1, % predicted	65.07 (20.61)	56.09 (28.12)	74.05 (5.43)	0.339†
FEV1/FVC, %	77.33 (5.61)	73.33 (5.51)	81.33 (0.58)	0.067†
Microbiology‡				
P. aeruginosa	1 (12.5)	1 (25.0)	0 (0.0)	1.000‡
mMRC	0.71 (0.76)	1.00 (1.00)	0.50 (0.58)	0.437†
Lab findings				
WBC count	8.27 (3.78)	10.43 (3.70)	6.10 (2.67)	0.106†
Neutrophil count, %	68.00 (6.46)	67.65 (8.39)	68.35 (5.17)	0.892†
Eosinophil count, %	1.76 (1.24)	1.70 (0.48)	1.82 (1.82)	0.899†
hs-CRP, mg/dl	1.72 (3.55)	2.94 (5.00)	0.51 (0.72)	0.374†

Data are presented as the mean (standard deviation) or number (%). Missing values were excluded listwise for each variable

BMI Body mass index, *COPD* Chronic obstructive pulmonary disease, *TB* Tuberculosis, *FVC* Forced vital capacity, *FEV1* Forced expiratory volume in 1 second, *NTM* Nontuberculous mycobacteria, *P. Aeruginosa, Pseudomonas aeruginosa, H. Influenzae, Haemophilus influenzae, S. Aureus, Staphylococcus aureus, K. Pneumoniae, Klebsiella pneumoniae, A. Baumannii, Acinetobacter baumannii,mMRC* Modified Medical Research Council, *WBC* Count, white blood cells count, *hs-CRP* High-sensitivity *C*-reactive protein

‡*Klebsiella pneumonia* and *Staphylococcus aureus* were identified in one patient

† P-values calculated using Student's t-test

‡ P-values calculated using Fisher's exact test

- P-values not applicable due to identical frequencies in both groups

the variance and component 2 accounting for 9.3%. Hierarchical analysis (Fig. 2e) showed that the expression of these 127 significant proteins increased (Fig. 2f) and decreased (Fig. 2g) in the AE groups. Detailed information on the 127 proteins was summarized in the Supplemental Table 2.

Of 127 differentially expressed proteins, 23 proteins showed more than 2-fold differences between the AE and stable status groups. Compared to the stable group, 18 proteins (TPI1, CRP, BPI, ORM1, PTPRE, S100A9, BPY2, TPM4, ERVFC1-1, CYS1, CLEC3B, S100A8, PSAT1, NDUFA10, MDGA1, SPRR3, ALDOA, and PSMB2) were significantly upregulated by more than 2-fold in the AE group (Supplementary Table 3). In contrast, 5 proteins (MUC5B, HSPE1, KLK13, IGHA1, and MUC5AC) were significantly downregulated by more than 2-fold in the AE group compared to the stable group (Supplementary Table 4). The volcano plot shows representative proteins whose expression was quantitatively increased (SPRR3, ORM1, S100A9, TPI1, and BPI) and decreased (MUC5B, HSPE1, KLK13, IGHA1, and MUC5AC) in the AE group compared to the stable group (Fig. 3; $|\log_2 \text{value}| \geq 0.5$). Overall, these differences indicate that increased neutrophilic inflammation and altered mucin and mucosal immunity are associated with the AE status of bronchiectasis.

Functional analysis

The 127 significant proteins obtained using the Student's t-test were analyzed via gene ontology (GO) using Metascape (Fig. 4). Twenty clusters enriched in all the 127 significant proteins are listed (accumulative hypergeometric p-values < 0.05). Neutrophil degranulation (R-HSA-6798695) was the most enriched pathway among those enriched in the 127 significant proteins, followed by C-type lection receptor (CLR) signaling (R-HSA-5621481).

Discussion

We conducted a proteomic analysis of BAL fluid using liquid chromatography-tandem mass spectrometry. Among the 1,577 proteins identified, 127 showed statistically significant differences between the patients with AE and those with stable bronchiectasis; we identified 18 proteins whose expression increased by more than 2-fold and 5 proteins whose expression decreased by more than 2-fold in the AE group compared to the stable group. GO analysis identified 20 functional clusters enriched in the significant proteins, with neutrophil degranulation being the most enriched pathway, followed by CLR signaling.

While previous studies have employed sputum analysis for proteomics research in bronchiectasis [12, 27, 28], this study is the first to utilize BAL fluid samples to molecularly characterize the AE of bronchiectasis. Here, we

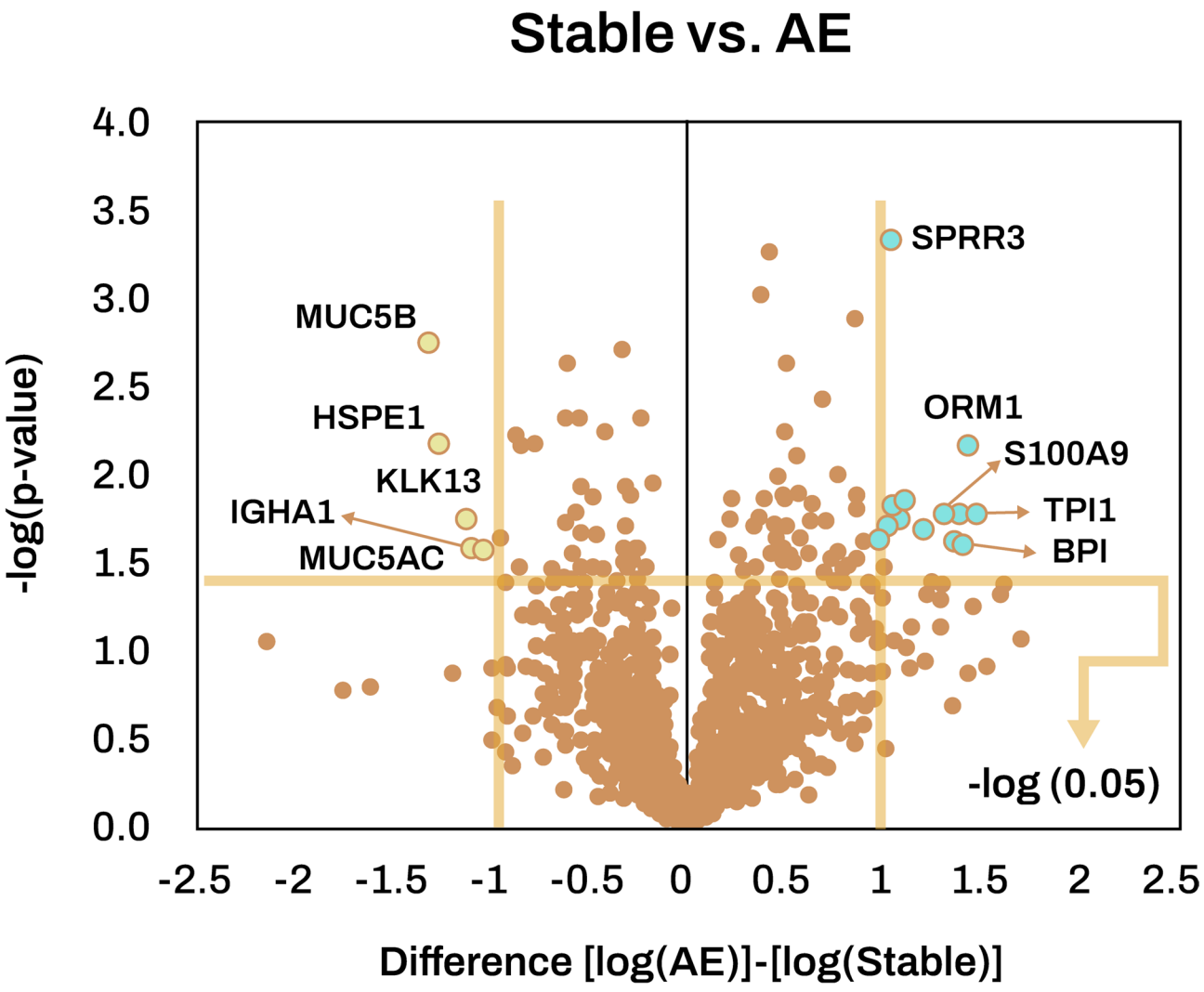

Fig. 3 Pathway enrichment analysis using the 127 significant proteins whose expression was altered between the AE and stable groups. The top 20 pathways with the most significant p-values (the -$\log_{10}$(p) values indicate that $p < 0.05$). Abbreviations: AE, exacerbation

found 127 proteins differentially expressed between the individuals in AE and stable status. Of these, 23 proteins showed marked differences demonstrating AE of bronchiectasis was associated with 18 upregulated proteins (TPI1, CRP, BPI, ORM1, PTPRE, S100A9, BPY2, TPM4, ERVFC1-1, CYS1, CLEC3B, S100A8, PSAT1, NDUFA10, MDGA1, SPRR3, ALDOA, and PSMB2) and 5 downregulated proteins (MUC5B, HSPE1, KLK13, IGHA1, and MUC5AC). Increased levels of CRP, S100A8, S100A9, BPI, and ORM1 suggest potentially ongoing neutrophil inflammatory conditions, and are involved in the regulation of inflammatory processes and immune responses in chronic inflammatory lung disease [29]. The decreased levels of MUC5B and MUC5AC suggest that their crucial role in maintaining and protecting the mucus layer in the airways is interrupted in inflammatory lung disease, and the decreased IGHA1 levels indicate that the protective role of immunoglobulin A in mucosal immunity is impaired [30, 31]. Also, reduced MUC5B/MUC5AC levels during exacerbations contrast with their elevated expression in chronic bronchitis [32]. This suggests that mucin depletion may reflect epithelial damage or dysregulated repair mechanisms during acute inflammation. Overall, these results indicate that neutrophilic inflammation and an altered mucin and mucosal immunity are closely linked to the AE status of bronchiectasis, which is in line with a recent study that analyzed the sputum proteomics associated with the AE of bronchiectasis [12].

Collectively, the differences in these inflammatory proteins observed show that neutrophil degranulation (R-HSA-6798695) was the most enriched pathway, reinforcing its important role in exacerbating bronchiectasis. Neutrophilic inflammation is the main mechanism of chronic airway inflammation in bronchiectasis [33]. In addition, neutrophil degranulation, including the release of neutrophil elastase and myeloperoxidase, plays

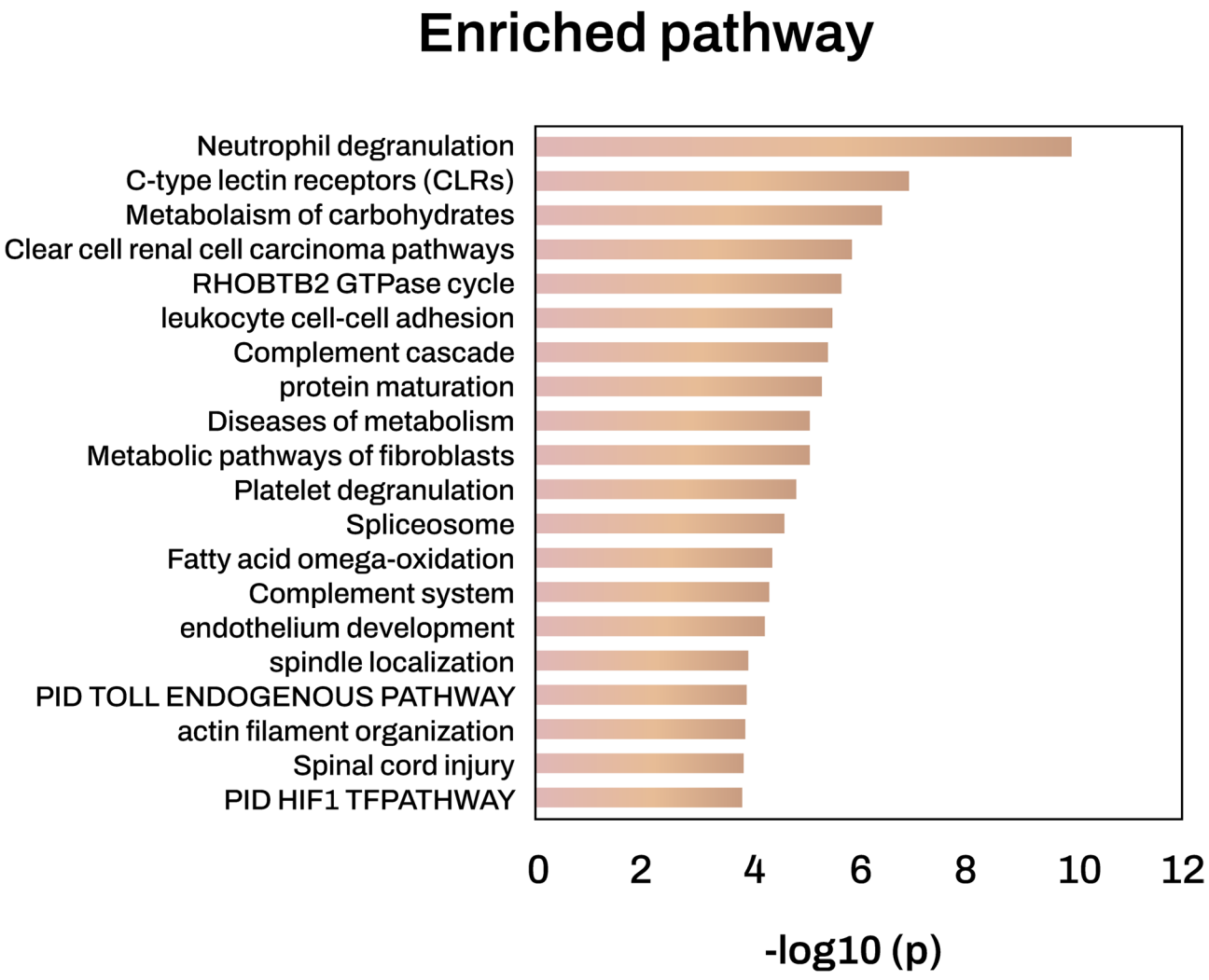

Fig. 4 Twenty-three proteins showing markedly differences between the AE status and stable status. Volcano plot was drawn to quantitatively compare the protein levels between the stable and AE groups. The dark blue and orange colors indicate the proteins whose levels were decreased and increased by more than two-fold, respectively (p-value < 0.05). Abbreviations: AE, exacerbation

a significant role in the AE of bronchiectasis [34]. Neutrophils release various inflammatory mediators, including proteases, reactive oxygen species, and cytokines. Although neutrophil elastase has a positive role in bacterial killing through phagocytosis, it also has negative roles, such as airway mucus obstruction, alveolar epithelial injury by degradation of the extracellular matrix proteins, activation of proinflammatory signaling, and compromise of innate immunity [35] These can exacerbate the symptoms of patients and contribute to the cycle of infection and neutrophilic inflammation that characterizes the disease. Our results support the role of neutrophilic inflammation in exacerbation of bronchiectasis. This finding aligns with recent therapeutic developments such as brensocatib, a dipeptidyl peptidase-1 inhibitor that suppresses neutrophil elastase activity and has demonstrated clinical benefit [36].

Additionally, we observed that the CLR pathway is an important pathway related to the AE of bronchiectasis. The CLR pathway is involved in shaping immune responses to various types of infections (bacterial, viral, and fungal). CLRs can induce phagocytic, endocytic, anti-microbial, anti-inflammatory, and proinflammatory responses to protect against infection, and have several functions according to the signaling motifs in the cytoplasmic domains. Moreover, the key roles of CLRs in allergy, autoimmunity, and homeostasis maintenance have been studied [37]. A previous study showed that the expression of mannose binding lectin (MBL), a soluble CLR, is associated with the severity and AE of bronchiectasis [38]. This finding supports our results, which suggest that C-type lectin receptors play an important role in bronchiectasis and its AE.

Our study has some limitations. First, the sample size ($n = 8$) is small and limits generalizability. The sample

size was chosen based on feasibility and the exploratory nature of proteomic analysis using BAL fluid, and the study was performed at only one center in Korea. Therefore, further studies are required to validate these findings. Second, due to the small number of participants, we could not perform subgroup analyses according to the AE endotypes of bronchiectasis and bacterial colonization. Third, our cross-sectional design precludes assessment of intra-individual proteomic changes. Longitudinal studies tracking BAL fluid before and after exacerbations are warranted. Fourth, a key limitation of our study is the inability to account for clinical heterogeneity across bronchiectasis endotypes. Exacerbations may arise from distinct mechanisms—including bacterial infection, neutrophilic inflammation, and eosinophilic or type 2 (T2) inflammation—which may exhibit divergent proteomic profiles. Although T2-high endotypes have been reported in up to 20–30% of bronchiectasis patients and are associated with increased exacerbation frequency, we did not perform T2 biomarker profiling due to the exploratory nature and small sample size of this study. Future investigations should incorporate endotype-specific analyses and integrate eosinophil-related markers to delineate mechanistically distinct exacerbation pathways. Finally, the absence of orthogonal validation of the identified differentially expressed proteins using independent techniques such as ELISA or immunoblotting. As this was an exploratory, hypothesis-generating proteomic study, our primary aim was to identify candidate proteins associated with bronchiectasis exacerbation. Future studies should validate these targets in larger patient cohorts using quantitative methods to confirm their clinical relevance and potential as biomarkers.

Conclusion

In conclusion, different BAL protein expression levels were associated with the AE status of bronchiectasis. Neutrophil degranulation and CLR pathways may play a role of the major pathways involved in the AE of bronchiectasis. Future studies with larger cohorts should be performed to corroborate these findings.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12890-025-03904-6.

Supplementary Material 1. Supplementary Table 1. List of 1,577 identified and quantified proteins from BAL proteome samples from bronchiectasis patients in stable and AE status. Abbreviations: AE, exacerbation. Supplementary Table 2. List of 127 significantly differentially expressed BAL proteins between bronchiectasis patients in stable and AE status. Abbreviations: AE, exacerbation. Supplementary Table 3. List of 18 proteins whose difference values increased by more than two-fold among the proteins that passed t test. Supplementary Table 4. List of proteins whose difference values decreased by more than two-fold among the proteins that passed t test.

Acknowledgements
Ethics statement: This study was approved by the Institutional Review Board of Chungbuk National University Hospital (No. 2021-10-07) and was conducted in accordance with the principles of the Declaration of Helsinki.

Authors' contributions
J.Y.L., J.Y., H.Y.K., Y.D., M-S.K., H.L., and B.Y. conceived the study and performed the experiments. J.Y.L., J.Y., H.L., and B.Y. wrote the manuscript and designed the diagrams. H.L., and B.Y. edited the manuscript. D.E.K., G.M. I-S.J., E-G.K., J.K.C. and B.Y. critically reviewed the paper.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declaration

Ethics approval and consent to participate
The study protocol was approved by the Institutional Review Board of the Chungbuk National University Hospital (application no. 2021-10-007).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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