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RESEARCH ARTICLE

Proteomics study of *Mycoplasma pneumoniae* pneumonia reveals the Fc fragment of the IgG-binding protein as a serum biomarker and implicates potential therapeutic targets

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Abstract **Macrolide and corticosteroid resistance has been reported in patients with *Mycoplasma pneumoniae* (MP) pneumonia (MPP). MP clearance is difficult to achieve through antibiotic treatment in sensitive patients with severe MPP (SMPP). SMPP in children might progress to airway remodeling and even bronchiolitis/bronchitis obliterans. Therefore, identifying serum biomarkers that indicate MPP progression and exploring new targeted drugs for SMPP treatment require urgency. In this study, serum samples were collected from patients with general MPP (GMPP) and SMPP to conduct proteomics profiling. The Fc fragment of the IgG-binding protein (FCGBP) was identified as the most promising indicator of SMPP. Biological enrichment analysis indicated uncontrolled inflammation in SMPP. ELISA results proved that the FCGBP level in patients with SMPP was substantially higher than that in patients with GMPP. Furthermore, the FCGBP levels showed a decreasing trend in patients with GMPP but the opposite trend in patients with SMPP during disease progression. Connectivity map analyses identified 25 possible targeted drugs for SMPP treatment. Among them, a mechanistic target of rapamycin kinase (mTOR) inhibitor, which is a macrolide compound and a cell proliferation inhibitor, was the most promising candidate for targeting SMPP. To our knowledge, this study was the first proteomics-based characterization of patients with SMPP and GMPP.**

Keywords severe *Mycoplasma pneumoniae* pneumonia; children; proteomics; Fc fragment of the IgG-binding protein; mechanistic target of rapamycin kinase inhibitor

Introduction

The clinical presentation of *Mycoplasma pneumoniae* (MP) pneumonia (MPP) includes pulmonary symptoms, such as bronchiolitis, pneumonia or necrotizing pneumonia, thrombosis, and extrapulmonary symptoms, such as encephalitis and hemolytic anemia. Persistent MP infection, airway obstruction, and airway remodeling have been reported in the airway epithelium of BALB/c mice and children with severe MPP (SMPP) [1–3]. Patients with MPP may develop life-threatening SMPP with respiratory failure, acute respiratory distress syndrome, and sequelae such as atelectasis and bronchiectasis caused by bronchiolitis/bronchitis obliterans (BO), which results from airway remodeling [2,4–7].

In consideration of the side effects of tetracyclines and quinolones, macrolides are used as first-line antibiotics for MPP treatment in young children. However, a high frequency of macrolide resistance has been reported; hence, MP clearance is difficult to achieve even when using sensitive antibiotics in patients with refractory MPP (RMPP) or SMPP [2]. The incidence of SMPP has recently increased, particularly in East Asia, thereby posing a

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serious threat to the health of children. Corticosteroids can effectively initiate the rapid improvement of clinical symptoms and chest radiographic findings; however, resistance to this drug has been reported in some patients with RMPP exhibiting severe presentations and serious radiological findings [8]. In the past 10 years, RMPP and SMPP cases have frequently occurred in northern China, especially at our hospital that has recorded a high rate of sequelae mainly due to patients with BO [2,4–6]. Thus, serum biomarkers and targeted drugs are urgently needed to monitor disease progression and prevent airway remodeling in patients with SMPP.

Numerous studies have identified biomarkers to detect RMPP or SMPP during the early stages. Several biomarkers, such as cytokines, have been used as markers to evaluate the severity of RMPP or SMPP in children. However, these biomarkers cannot completely predict severity or sequelae, which limits their clinical application. Proteomics has emerged as a powerful method to investigate novel diagnostic and therapeutic targets. With rapid advances in mass spectrometry technology, the consistency of peptide identification and protein sequence coverage in complex biological samples has been substantially improved. Label-free proteomics technologies are considered as a superior method because of their high proteome coverage and labeling efficiency, particularly for obtaining information that cannot be accessed using 2D electrophoresis. These methods are widely used in quantitative proteomics.

In this study, label-free proteomics analysis was initially conducted in children with GMPP and SMPP to identify differentially expressed proteins. The Fc fragment of the IgG-binding protein (FCGBP) was found to have the highest differential expression, and this finding was verified by the ELISA-based quantification of expression levels. Connectivity map (CMap) was used to identify potentially useful drugs for the treatment of patients with SMPP and to further explore the mechanism of SMPP. To the best of our knowledge, this study is the first to employ this approach to characterize the progression of MP infection-related diseases.

Materials and methods

Clinical samples

This study enrolled 89 patients with MPP (38 with GMPP and 51 with SMPP). Among them, 4 (2 with GMPP and 2 with SMPP) were analyzed using label-free proteomics analyses, and the remaining 85 (36 with GMPP and 49 with SMPP) were tested using ELISA. Serum samples were collected from patients between January 2016 and January 2017 at Beijing Children's Hospital, Capital Medical University, China, stored, and frozen at −80 °C. Written informed consent was obtained from the caregivers of all children prior to the experiment. This study was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University (No. 2017-23).

Diagnostic criteria

MPP was diagnosed based on the following criteria: (1) clinical presentation (fever and cough); (2) chest imaging with infiltrates; and (3) serum anti-MP IgM titer $\geqslant$ 1:256 (double testing) or a fourfold increase in titers in acute and convalescent serum specimens. Real-time PCR for MP DNA in throat swabs was positive during the acute stage in all patients. SMPP was defined as MPP with the following symptoms: (1) pulse oxygen saturation in room air $\leqslant$ 92% and (2) involvement of $\geqslant$ 2/3 pulmonary lobes. No other respiratory tract pathogens were detected in the enrolled patients. All participants were previously healthy and had received azithromycin therapy prior to sample collection.

Proteomics mass spectrometry

Peripheral blood samples were collected from patients with MPP and then centrifuged for 10 min at 2000 × *g* to obtain serum samples. Same-group (GMPP or SMPP) samples were pooled at ratio of 1:1 for the label-free quantitative proteomics analyses. In brief, 1 μL of sample from the pooled serum of each group was used for 10% SDS-PAGE (Fig. S1). After separation, staining, destaining, and in-gel digestion, the peptides were resolved and subjected to mass spectral analyses (Thermo, Q-Exactive). MaxQuant was applied to process the raw mass spectrometry data, and the peptides were searched against the UniProt protein database with the following parameters: variable modifications: acetyl (protein N-term), oxidation; enzyme: trypsin/P; max missed cleavages: 2; MS/MS: 20 ppm; and protein FDR = 0.01. Other parameters were set to default. Proteins with false hits labeled by MaxQuant, including "potential contaminant," "reverse," and "only identified by site," were excluded. Only the proteins detected in both groups were included in the subsequent analyses. Finally, 276 proteins satisfying the above criteria were identified. Label-free quantification (LFQ) intensity was used to represent the protein expression levels. The raw data are publicly available from the integrated Proteome Resources Center (iProX) with the accession number IPX0001738000.

Functional enrichment analysis

"ClusterProfiler" [9] R package was used for the enrichment of Gene Ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Enrichment significance was estimated using the default setting and 1000 permutations.

Benjamini–Hochberg-adjusted P values < 0.05 were considered statistically significant. The KEGG pathway overview was implemented using the "pathview" R package [10].

ELISA

The targeted differentially expressed proteins were identified, and FCGBP was found to have the highest differential expression. ELISAs were then conducted following the manufacturer's instructions (Aviva Systems Biology, San Diego, CA, USA) to verify the circulating protein concentrations of FCGBP in patients with SMPP and GMPP. The antibody specific for FCGBP was pe-coated onto a 96-well plate. The serum sample was added to the wells, incubated, and removed. Similar procedure was subsequently adopted for biotinylated detector antibody for FCGBP and avidin-peroxidase conjugate. Enzymatic reaction occurred after the TMB substrate was added, and the O.D. absorbance at 450 nm was used to quantify the amount of FCGBP.

Prediction of the transcription factors (TFs) regulating FCGBP

Cistrome Data Browser [11] was used to predict potential TFs that regulate the FCGBP-targeted protein. This toolkit is a user-friendly web server for querying, exploring, and visualizing ChIP-seq and chromatin accessibility data. In consideration of the limited ChIP-Seq data publicly available in BEAS-2B cells, an epithelial cell line isolated from normal human bronchial epithelium, potential TFs for FCGBP in all available cells were determined within a 100 kb distance from the transcription start site to include enhancer-type TFs.

CMap analysis

Drug prediction, mechanism of action (MoA), and drugtarget analyses were performed using CMap tools [12]. CMap integrates gene expression signatures, drugs, and disease states and is widely used to predict potentially useful drugs and investigate the internal mechanism and interrelationships of drugs, thus providing information for clinical trials. The query and touchstone functions of the CMap website were used for drug predictions, MoA, and drug–target analyses. Dysregulated genes between GMPP and patients with SMPP were subjected to a query tool. The "bitr" function of the R package "cluster Profiler" was implemented to convert the UniProt accessions into gene symbols. The R package "Complex Heatmap" [13] was used to visualize MoA and drug–target analysis results. Interaction relationships between the target genes of selected compounds were analyzed and visualized using the STRING.

Statistical analysis

All statistical analyses were performed using R version 3.6.0. T test was used to detect significant differences between patients with SMPP and GMPP for continuous variables, and Fisher's exact test was applied for categorical variables. Proteins with an absolute value for $\log_2$ fold change ($\log_2$FC) > 0.5 between patients with GMPP and SMPP were considered differentially expressed proteins. A P value < 0.05 or Benjamini–Hochberg-adjusted P value < 0.05 when executing multiple comparisons was considered statistically significant.

Results

Characteristics of patients with GMPP and SMPP

Table 1 shows the detailed characteristics of patients enrolled in the label-free proteomic study. Among them, one patient with SMPP had hydrothorax. Necrotizing pneumonia, an uncommon severe complication of pneumonia, was observed in the late stage. All the patients involved in ELISA were treated with azithromycin, corticosteroids, and bronchoscopy lavage therapy and followed up for at least 3 months. Table 2 shows their clinical characteristics (detailed in Table S1). The mean C-reactive protein (CRP) levels at admission were 23.69 and 82.31 mg/L in patients with GMPP and SMPP, respectively. Chest imaging showed consolidation with a high density in $> 1/2$ pulmonary lobes in all patients with GMPP and $> 2/3$ lobes in all patients with SMPP. In the early stage of the disease, two patients with GMPP (5.6%) and 39 patients with SMPP (79.6%) showed a mucus plug/thick mucus secretion on bronchoscopy, and 18 (36.7%) patients with SMPP showed airway mucous necrosis. In the late stage, bronchoscopy revealed airway deformation due to airway remodeling in 24 patients with SMPP (14 with BO). No sequelae were observed in the patients with GMPP. Significant difference in airway damage was found between the two groups ($P < 0.05$).

Identification of differentially expressed proteins and distinct biological processes between patients with GMPP and SMPP

A total of 130 differentially expressed proteins were identified, of which 61 were upregulated in patients with GMPP and 69 were upregulated in patients with SMPP. Several inflammatory markers, such as CRP, serum amyloid A1 (SAA1), and serum amyloid A2 (SAA2), were upregulated in patients with SMPP (Fig. 1A). Among these differentially expressed proteins, FCGBP was one of the most altered proteins and had the highest fold change. Biological processes such as humoral immune response,

Table 1 Detailed clinical characteristic of children enrolled in proteomics analysis

Patients	Age	Gender	Duration of fever (day)	Duration of cough (day)	WBC ($\times 10^9$/L)	NEUT (%)	CRP (mg/L)	D-dimer (mg/L)	LDH	Radiology	Treatment	Following clinical symptoms
GMPP												
1	9y3m	Male	5	5	7.6	63.3	8	0.146	167	Right upper shadow	Azithromycin	–
2	3y11m	Female	7	7	5.7	32.3	25.3	0.378	308	Right upper shadow	Azithromycin	–
SMPP												
1	9y4m	Male	20	20	4.49	78.2	72	7.355	298	Pulmonary consolidation at right upper, hydrothorax	Azithromycin, glucocorticoid	Necrotizing pneumonia
2	12y2m	Female	8	8	10.9	80.9	277	1.058	643	Pulmonary consolidation at large scope	Azithromycin, glucocorticoid	–

WBC, white blood cell; NEUT, neutral; CRP, C-reactive protein; LDH, lactate dehydrogenase; "y" in line "Age", years; "m" in line "Age", months.

Table 2 Clinical characteristics of patients enrolled in ELISA

	Overall (n = 85)	GMPP (n = 36)	SMPP (n = 49)	P
Gender, n (%)				
Female	34 (40.0)	15 (41.7)	19 (38.8)	0.964
Male	51 (60.0)	21 (58.3)	30 (61.2)	
Age (mean (SD))	7.72 (2.43)	7.97 (2.43)	7.53 (2.43)	0.413
CRP (mean (SD))	57.48 (47.62)	23.69 (17.54)	82.31 (47.52)	<0.001
Bronchial occlusion, n (%)				
No	71 (83.5)	36 (100.0)	35 (71.4)	0.001
Yes	14 (16.5)	0 (0.0)	14 (28.6)	
Mucus plug, n (%)				
No	44 (51.8)	34 (94.4)	10 (20.4)	<0.001
Yes	41 (48.2)	2 (5.6)	39 (79.6)	
Airway mucous necrosis, n (%)				
No	67 (78.8)	36 (100.0)	31 (63.3)	<0.001
Yes	18 (21.2)	0 (0.0)	18 (36.7)	
Airway deformation, n (%)				
No	61 (71.8)	36 (100.0)	25 (51.0)	<0.001
Yes	24 (28.2)	0 (0.0)	24 (49.0)	

acute inflammatory response, protein activation cascade, and receptor-mediated endocytosis were enriched in patients with SMPP (Fig. 1B, Table S2). Meanwhile, hydrolase activity, peptidase activity, and plasma lipoprotein particles were enriched in patients with GMPP (Fig. 1C, Table S3). KEGG analysis indicated uncontrolled inflammation in the SMPP group as evidenced by the enrichment of pathways, such as complement and coagulation cascades, *Staphylococcus aureus* infection, IL-17 and HIF-1 signaling pathways, and platelet activation (Fig. 1D, Tables S4–S5). The complement and coagulation cascade pathways were enriched in patients with GMPP and SMPP. Complement activation helps recruit inflammatory and immunocompetent cells and clears extraneous pathogens. Blood coagulation regulates blood pressure and sodium homeostasis. Fig. 1E shows an overview of alterations in complement and coagulation cascade pathways between GMPP and SMPP. Many components of the coagulation system, including coagulation factors such as F2, F5, F9, F13, and fibrinogen, were upregulated in patients with SMPP. Meanwhile, complement components C2, C4, and clusterin were altered in patients with GMPP.

FCGBP is a novel indicator for predicting SMPP progression

ELISA was used to determine the serum expression levels of FCGBP in patients with GMPP and SMPP to explore its possible application as a serum marker to indicate the

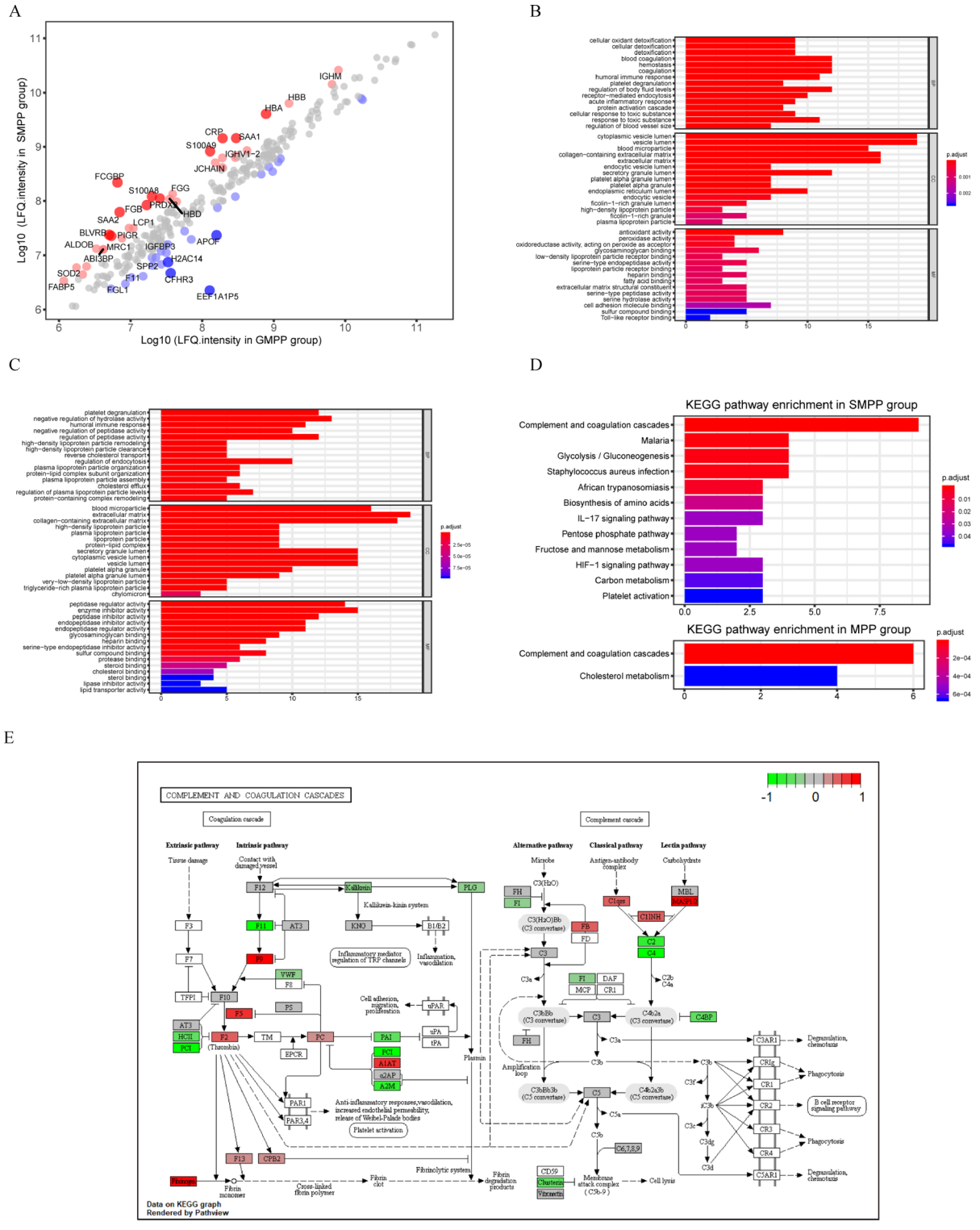

Fig. 1 Biological differences between general *Mycoplasma pneumoniae* pneumonia (GMPP) and severe *Mycoplasma pneumoniae* pneumonia (SMPP). (A) 2D scatter plot of the proteins identified in patients with GMPP and SMPP. Proteins with $\log_2$FC > 0.5 were considered to be differentially expressed proteins and are shaded in blue or red. Corresponding gene symbols of proteins with $\log_2$FC > 1.5 are labeled in the plot. The label-free quantification (LFQ) intensity represents the protein quantification level. (B, C) Gene ontology enrichment analysis results of the dysregulated genes enriched in patients with SMPP (B) and GMPP (C). Top 15 GO terms with the most significant enrichment results for each category (biological process, molecular function, and cellular component). (D) Kyoto Encyclopedia of Genes and Genomes analysis results for the dysregulated genes enriched in patients with SMPP (top) and GMPP (bottom). All enrichment terms that reached significance are shown. (E) Overview of the complement and coagulation cascade pathways. The colored boxes (green, gray, and red) are the identified proteins in these pathways. The color intensity represents the corresponding $\log_2$FC level; and increase in color intensity indicates an increase in enrichment in patients with SMPP (red) and GMPP (green).

presence and progression of MPP. On the 1st day of admission, patients with SMPP showed higher FCGBP expression than patients with GMPP with twofold changes (Fig. 2A, $\log_2$FC = 1, P = 0.016). At the late stage of the disease, patients with SMPP exhibited significantly higher FCGBP expression than patients with GMPP (Fig. 2B, $\log_2$FC = 2.31, P = 0.0059). The expression level of serum FCGBP exhibited a decreasing trend in patients with GMPP during the disease course but an increasing trend in the SMPP group after appropriate treatment (Fig. 2C and 2D). Severe sequelae such as BO tended to occur in patients with SMPP. In addition, the ROC curves of late-stage patients revealed the ability of FCGBP to distinguish SMPP from GMPP (Fig. 3A, AUC = 0.833). These data suggested that FCGBP may be a promising indicator for predicting SMPP progression, and patients who maintain high serum FCGBP levels during treatment may have worse prognosis compared with those with low levels.

Potential TFs regulating FCGBP expression

The mechanism underlying the dysregulated expression of FCGBP remains poorly understood. Given that TFs control appropriate gene expression from DNA to mRNA, FCGBP was subjected to the Cistrome Data Browser toolkit to predict potential TFs (details can be found in the section of "Materials and methods"). Fig. 3B shows the top 20 identified TFs with the highest regulatory potential scores. Among them, many factors were correlated with T cell differentiation, such as GATA3, and with inflammation, such as RELA. These results suggested that these TFs might regulate FCGBP expression, thus promoting the development and increasing the severity of SMPP.

Identification of potential SMPP therapeutic drugs including mTOR inhibitors

CMap, the world's largest perturbation-driven gene expression data set, was used to determine perturbations in expression signatures and identify candidate therapeutic drugs. The connectivity score is a holistic quantification index evaluating the similarity between the query signature and reference metagenes. The top 10 compounds with the most positive and most negative connectivity scores are

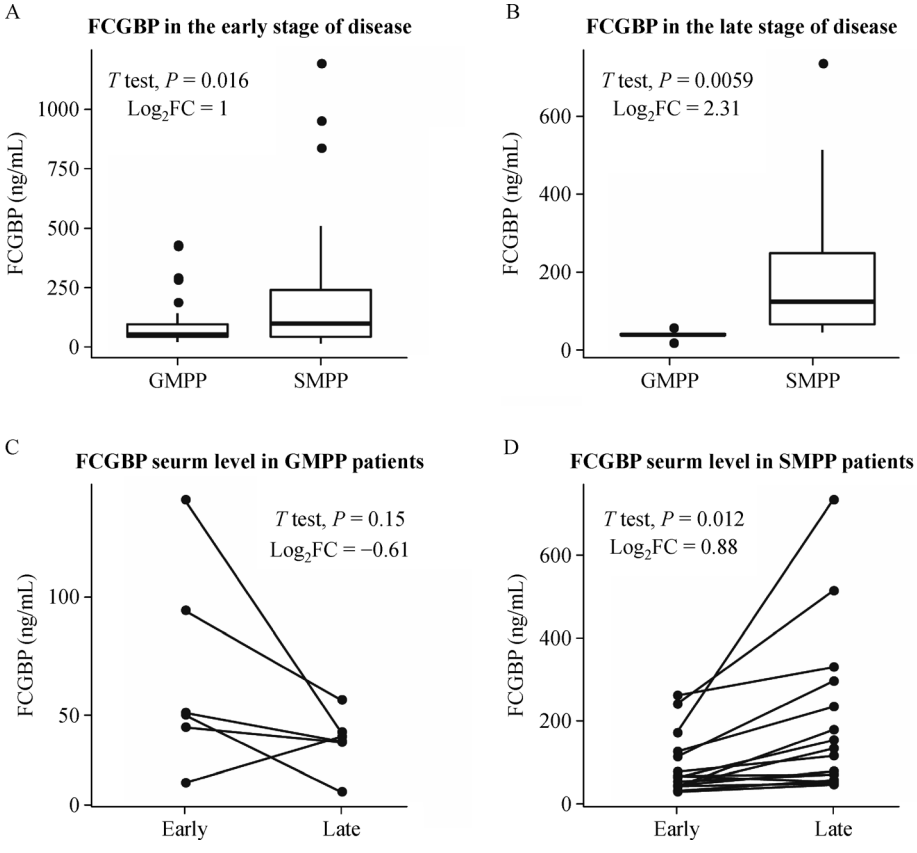

Fig. 2 Fc fragment of the IgG-binding protein (FCGBP) is a promising indicator for the progression to severe *Mycoplasma pneumoniae* pneumonia (SMPP). The serum concentration of FCGBP identified by ELISA in patients with general *Mycoplasma pneumoniae* pneumonia (GMPP) and SMPP on the 1st day of admission (A) and 5–7 days after admission (B). Statistical significance level of comparisons and fold change level (SMPP vs. GMPP) are indicated in the text. (C) Pairwise comparison of FCGBP serum concentrations in the GMPP group for treatment-naïve patients on the 1st day of admission and after 5–7 days of admission. (D) Pairwise comparison of FCGBP serum concentrations in the SMPP group for the patients on the 1st day of admission and after 5–7 days of admission. The statistical significance level of pairwise comparisons is indicated in the text.

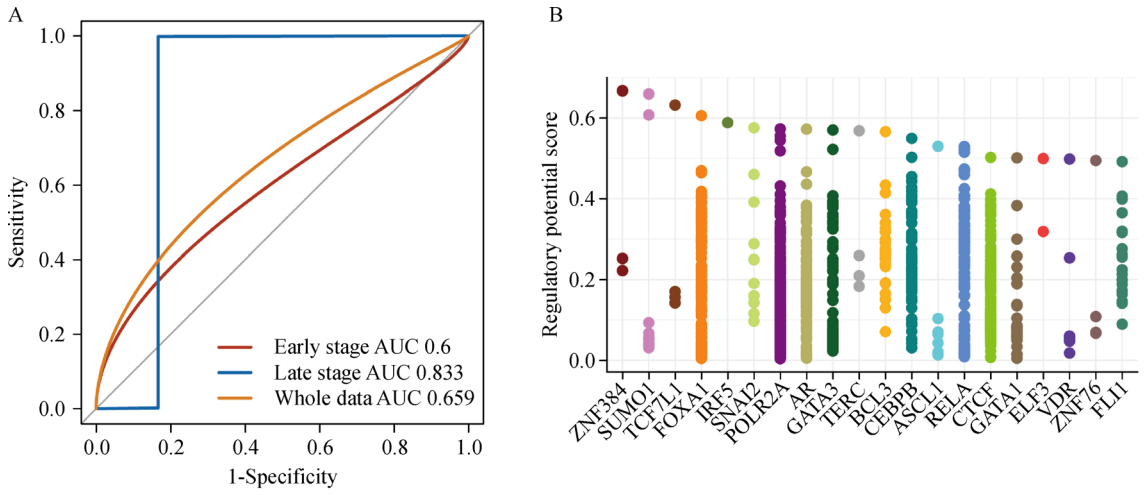

Fig. 3 Potential transcription factors (TFs) that regulate the expression of IgG-binding protein Fc fragment. (A) Predictive value of FCGBP serum levels in the early, late, and all stages. (B) Top 20 TFs with the highest regulatory potential scores. The regulatory potential score estimates the possibility that a factor can regulate a gene. Factor names are placed on the *x*-axis. Dots on the same vertical line with the same color represent the same factor. Each dot represents ChIP-Seq data.

shown in Fig. 4A. Compounds with connectivity score of < -70 were selected as candidate drugs (complete results are shown in Table S6). Finally, 25 drugs were selected for further analyses. CMap MoA analysis of these 25 drugs showed 27 potential underlying mechanisms (Fig. 4B). In addition to commonly known drug mechanisms such as anti-inflammatory factors and acetylcholine receptor antagonists, mTOR inhibitors were also identified. Gene target analysis of these 25 drugs revealed 54 target genes (Fig. 4C). Although no overlap was found between these target genes, protein–protein interaction analyses indicated a high connection between them (Fig. 4D).

Discussion

This study provides a biological picture of GMPP and SMPP at the proteomic level. Human cells mobilize the immune system to eradicate pathogens such as MP when they first encounter foreign pathogens. However, uncontrolled inflammation may occur when patients are in poor condition. Distinct biological processes were observed between patients with GMPP and SMPP. mTOR might play an important role in MPP progression. FCGBP, a promising serum indicator of SMPP, was identified in this work. However, this serum needs large cohort verification for clinical usage.

With the development of new techniques, high-throughput sequencing has been increasingly used in MP studies. Lluch–Senar *et al.* performed a comparative "-omics" analysis and reported more toxigenic type 2 strains than type 1 strains for MP because of the high expression levels of CARDS toxin [14]. Using the RNA sequencing of bronchoalveolar lavage fluid, Gao *et al.* found 810 differentially expressed genes in the MPP group compared with the control group, suggesting that NK and $CD8^+$ T cells are over-activated and proliferated in children with MPP [15].

Proteomics has been increasingly used to identify the biomarkers of infectious diseases and provide a feasible method for the large-scale screening of SMPP-related proteins, thus enhancing our understanding of SMPP pathogenesis. To date, only two studies have conducted the proteomics analysis of MPP [16,17]; one study used serum samples from five children with RMPP, five children with non-RMPP, and five healthy children, and the other employed plasma from children with MPP, infectious disease controls, and healthy controls. Only a few reports have focused on the proteomics analysis of SMPP. To the best of our knowledge, the present study is the first to conduct proteomics analysis on patients with GMPP and SMPP to identify markers for SMPP progression.

A total of 130 differentially expressed proteins were initially identified between children with GMPP and SMPP by using label-free quantitative analysis. The complement and coagulation cascade pathways were enriched in all patients, and this finding could be attributed to the activation extent for various pathways. Given that the moderate activation of complement and coagulation cascade pathways assists in clearing microbial intruders, the host (patients with GMPP) must have attempted to clear pathogens and maintain homeostasis by activating these pathways. The dysregulation of the acute phase involving a cytokine storm can result in severe clinical symptoms such as acute lung injury [18], hypercoagulable states, and even thrombosis [4]. The disorder of the complement and coagulation cascade pathways might play a vital role in SMPP incidence. In addition, collagen-

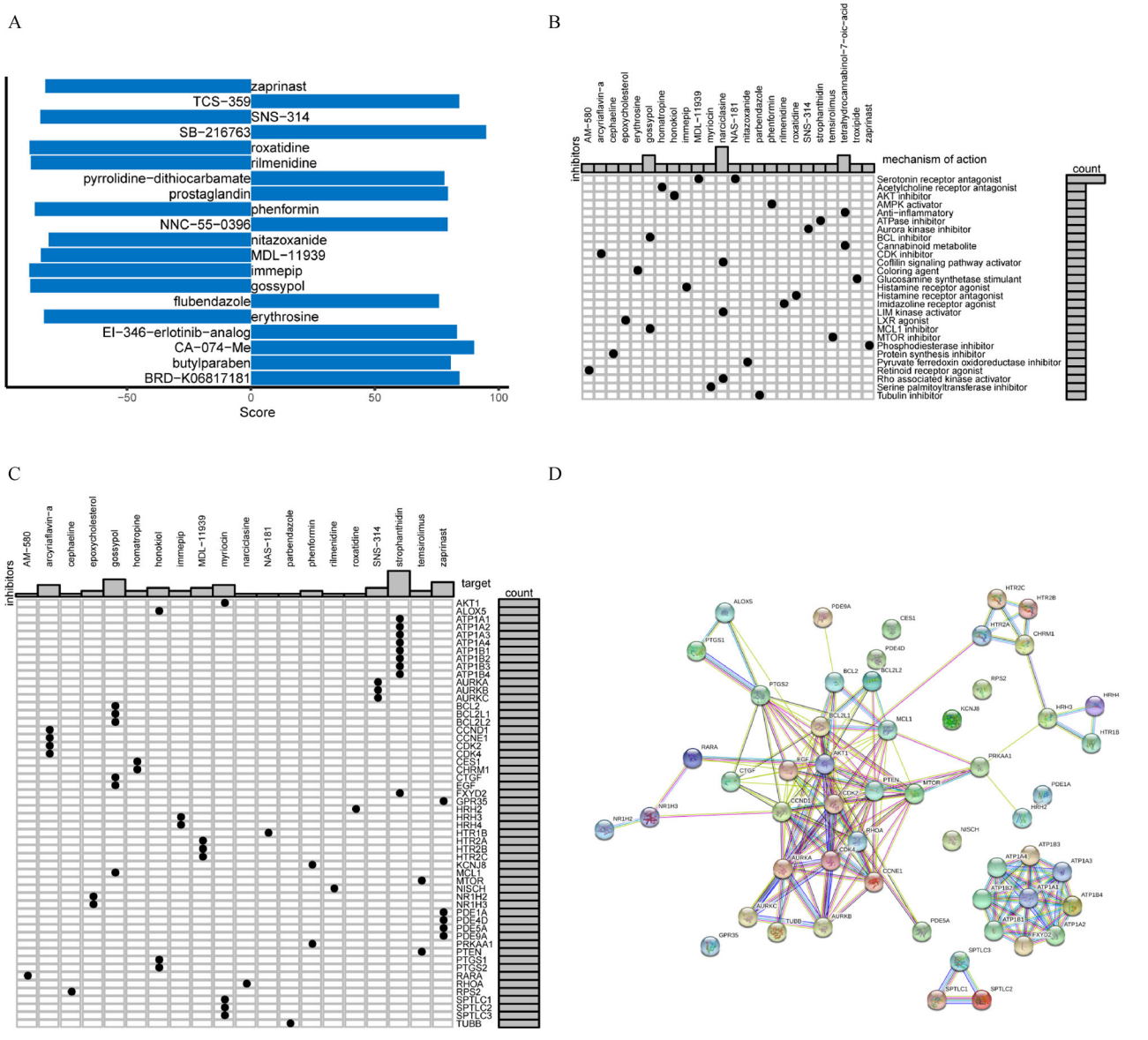

Fig. 4 Promising therapeutic drugs that inhibit progression to severe *Mycoplasma pneumoniae* pneumonia. (A) A bar graph of candidate perturbations inferred from connectivity map analyses. The top 10 compounds with the most positive and most negative connectivity scores are shown. (B) Connectivity map (CMap) mode-of-action analyses of the 25 selected drugs with connectivity scores < -70. Potential mechanisms and inhibitors are placed in the right and upper panels. The perturbations' modes of action are indicated by black dots. (C) CMap gene target analyses of the 25 selected drugs. Identified target genes and inhibitors are placed in the right and upper panels. The perturbations' target genes are indicated by black dots. (D) Protein–protein interaction network of the target genes inferred from CMap gene target analysis. The relationships between these proteins are inferred from STRING.

containing extracellular matrix and platelet degranulation were enriched in all patients (Fig. 1B and 1C). KEGG analysis revealed the enriched platelet activation in patients with SMPP (Fig. 1D), which could be explained by the altered airway remodeling and the extent of platelet activation. The typical characteristics of SMPP include an excessive immune response, a hypercoagulable state, thrombosis, and subsequent airway remodeling, such as airway stenosis and BO [4]. Platelets are key mediators of inflammatory responses and cooperate with coagulation pathway to activate innate immune cells [19]. Activated platelets may regulate pulmonary immune defenses, inflammatory injury [20], and immunothrombosis [21] and are necessary for airway wall remodeling [22]. Therefore, the disorder of platelet activation might play a vital role in SMPP.

Among the identified differentially expressed proteins, including CRP and SAA, FCGBP was one of the most altered proteins and had the highest fold change. As a gel-like component of the mucosa, this protein possibly

maintains the mucosal structure and can covalently bind and cross-link mucus proteins via its autocatalytical cleavage of von Willebrand D domain [23]. The differential expression of FCGBP has been reported in gallbladder, prostate, thyroid, lung [24], colon, and ovarian cancer. This protein is sometimes highly expressed in the serum of patients with autoimmune diseases [25]. FCGBP has been postulated to trap HIV-1-antibody complexes at mucosal surfaces [26]. *FCGBP* is the most upregulated early defense gene in catfish skin after microbial infection [27], and its expression was assumed to be modulated by HPV infection [28]. FCGBP binds to trefoil factor family 3 (TFF3) to form a heterodimeric complex [29]. TFF3 shows lectin activity, binds to bacterial glycans such as the lipopolysaccharide of *Helicobacter pylori* (*H. pylori*), and exhibits potential antibacterial properties [30,31]. Therefore, FCGBP is considered a component of the first-line response in innate immunological defense because it critically regulates pathogen attachment and disease progression on the mucosal surface, thus participating in cell protection and anti-inflammation in tissues.

In this study, patients with SMPP showed higher FCGBP expression than patients with GMPP in the early stage, but its diagnostic ability is less optimistic (Fig. 4A, AUC = 0.6). This finding may be due to the acute stage of the disease or its relative severity in patients with GMPP who had consolidation in $> 1/2$ pulmonary lobes on chest imaging at our hospital. In the late stage, patients with GMPP did not require additional FCGBP; hence, its expression level was reduced. However, for patients with SMPP, airway damage was not relieved even when the CRP level was restored to baseline and the body temperature returned to normal. Airway deformation such as BO also occurred. This finding suggested the pro-inflammation and pro-remodeling effect of airway epithelial cell and fibroblast proliferation, airway remodeling, and FCGBP. The AUC at the late stage reached 0.833, implying that FCGBP could be a biomarker for MPP progression or airway remodeling in MPP. Owing to the relatively small proportion of late-stage patients in our enrolled cohort (85 patients in ELISA studies and only 22 patients with multi-time sampling), the ROC curve of all samples, including early and late stages, was below 0.7. A well-designed and large cohort is needed in a future randomized controlled trial prior to clinical application.

FCGBP is a key regulator of transforming growth factor β1 (TGF-β1)-induced epithelial-mesenchymal transition (EMT) in gallbladder cancer [32]. TGF-β is a platelet-granule constituent [21], and airway remodeling is consistent with EMT in MP infection [1]. Thus, we propose that FCGBP and platelet activation are important in airway remodeling in SMPP. Among the identified TFs, TCF7L1 ranked in the top three. This gene is a member of the T cell factor/lymphoid enhancer factor family of TFs and is involved in the Wnt signaling pathway to facilitate the precise differentiation of human stem cells [33,34]. A study based on genome-wide associations for smallpox vaccine responses confirmed that TCF7L1 is critical for lymphocyte IFNγ production or cytotoxic T cell function [35], revealing its important role in regulating inflammatory responses and eradicating foreign pathogens. In addition, numerous studies have linked the activation of CEBPB and RELA with immune and inflammatory responses, suggesting their involvement with FCGBP during SMPP development. RELA is a subunit of NF-κB, a major transcriptional regulator of genes involved in survival, proliferation, and inflammation. Its activation is involved in MP infection [36].

Anti-inflammatory treatment involving glucocorticoids is important for SMPP treatment. However, glucocorticoid resistance has been reported in many patients with SMPP [8]. Compared with that in patients with SMPP, steroid binding was enriched in patients with GMPP (Fig. 1C). Hence, this phenomenon might predict a good prognosis. Many patients with SMPP miss their best glucocorticoid therapeutic opportunity in the early stage of disease. Glucocorticoids also have side effects, such as osteoporosis and an increased risk of secondary infection. Patients who develop SMPP frequently experience respiratory failure, massive pleural effusion, atelectasis, necrotizing pneumonia, BO, and life-threatening conditions. Therefore, developing drugs that prevent GMPP from progressing to SMPP is of great importance. In our study, the predicted 25 drugs may provide an opportunity for the early and effective treatment of this disease. The mTOR inhibitor temsirolimus was also identified. Vascular endothelial growth factor (VEGF) released by endothelial cells induces angiogenesis, and its elevated expression has been reported in some patients with MPP [37] and SMPP (unpublished data). The mTOR inhibitor temsirolimus, an immunosuppressive agent and a macrolide compound, was isolated from *Streptomyces hygroscopicus* in a soil sample from Easter Island in 1975. This substance may effectively suppress inflammation and angiogenesis, including VEGF levels, and block T cell activation and proliferation. Temsirolimus is the prodrug of sirolimus and is converted into sirolimus *in vivo*. Sirolimus may be useful for the treatment of acute lung injury [38] because it decreases the airway remodeling caused by TGF-α-induced/epidermal growth factor receptor-mediated signaling [38]. Given that the TOR pathway regulates morphogenesis and responses to host cells in the fungal pathogen *Candida albicans* [39], it may also control the responses to host cells in MPP. Low sirolimus dosages have a good profile for safety and tolerability in pediatric patients [40]. Considering the evidence on angiogenesis, excessive inflammatory responses, T cell proliferation, and airway remodeling in SMPP, we suspect that sirolimus, a macrolide antibiotic, is a promising drug for SMPP treatment. Further investigations of the sensitivity of MP to this drug, elucidation of the

mechanisms of mTOR inhibitors for SMPP treatment, and randomized clinical trials are needed to promote the clinical usage of sirolimus.

Conclusions

This study conducted the first proteomics analysis on patients with SMPP and GMPP. The results suggested that FCGBP is a new serum biomarker for monitoring SMPP progression. Sirolimus, an mTOR inhibitor, is a promising targeted drug for SMPP treatment.

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Compliance with ethics guidelines

Jinrong Liu, Rongfang Shen, Lin Feng, Shujun Cheng, Jun Chen, Ting Xiao, and Shunying Zhao declare that there are no conflicts of interest. Written informed consent was obtained from the caregivers of all children before starting the study. This study was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University (No. 2017-23).

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