Identification of candidate biomarkers for severe adenovirus community-acquired pneumonia by proteomic approach

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ABSTRACT

Introduction: Worldwide, Human adenoviruses (ADV) cause a significant portion of childhood mortality. The severity of ADV Community-acquired Pneumonia (CAP) can be assessed by clinical features, but the rapid and accurate diagnostic biomarkers are still lacking. Candidate biomarkers for severe ADV CAP are to be screened and the different protein expression levels associated with pediatric ADV CAP may help assess the severity of ADV CAP for the pediatricians to make early intervention.

Methods: In our study, serum samples from healthy controls, patients with ADV CAP, streptococcus pneumonia (SP) and respiratory syncytial virus (RSV) infection were collected. Differently expressed proteins (DEPs) were detected by iTRAQ-based mass spectrometry. Gene Ontology and Pathway Enrichment analysis of DEPs were performed by Cytoscape. The protein interaction network for the identified proteins was constructed by String.

Results: The results showed that 119 DEPs in mild ADV CAP and 148 DEPs in severe ADV CAP were identified, compared with healthy children. Four proteins (Protein S100-A9 (S100A9), Protein S100-A8 (S100A8), Leucine aminopeptidase III (LAP3), and Apolipoprotein A-IV (APOA4)) were validated by Western blot, and results indicated that the expression levels of these four proteins were consistent with the proteomic analysis. LAP3 was the most significantly up-regulated protein in severe ADV CAP compared to mild group. In addition, LAP3 was the most significantly up-regulated protein in severe ADV CAP comparing with SP CAP infection and RSV CAP infection.

Conclusion: Our findings identified LAP3 protein as a potential diagnostic biomarker which can assess the severity of ADV CAP.

1. Introduction

Human adenovirus (ADV) is one of the most important causes of community-acquired pneumonia (CAP), particularly in the pediatric population [1]. ADV-associated respiratory infection is usually mild, self-limited, and indistinguishable from other viral respiratory infections [2]. However, ADV infection can cause severe CAP with high mortality and permanent lung damages in some patients [3]. Long-term respiratory sequela of severe ADV CAP include bronchiolitis obliterans, hyperlucent lung, and bronchiectasis [4].

Presently, the diagnosis of ADV was based on immunofluorescence, viral cultures and PCR identification [5]. The severity of ADV CAP was depended on the clinical characteristics and pulmonary imaging since the rapid and accurate diagnostic biomarkers are lacking [6]. The gold standard is viral cultures, which might be insensitive for certain samples (e.g., blood) and more time-consuming to provide results in the acute stage [5]. Biopsy of involved tissues may reveal ADV nuclear inclusions [7] and immunohistochemical stains may identify the ADV hexon antigen in tissues [8]. However, lung tissue specimens from children with ADV CAP are rarely available. PCR of ADV DNA in plasma, urine, or other
clinical specimens and rapid antigen detection kits for ADV are currently the most frequently used approaches for the diagnosis [7, 9]. However, these methods can only diagnose ADV infections, but are not able to accurately assess the severity of the ADV CAP [10].

Previous studies showed that the severity of ADV infection is significantly correlated with serotype, which had not been routinely performed [11]. Various acute phase reactants have been used to assess the severity of CAP, including procalcitonin (PCT), cytokines, C-reactive protein (CRP), and lactate dehydrogenase (LDH). However, their predictive value varied among different studies [12, 13]. It is urgently needed to explore novel diagnosis biomarkers in the acute stage of severe ADV CAP. Here, we aim to screen biomarkers to diagnose severe ADV CAP by using isobaric tags for relative and absolute quantitation (iTRAQ) quantitative proteomics technique, which is used widely for diagnosis and therapy [14] because of its high proteome coverage and labeling efficiency [15]. The different protein expression levels associated with pediatric ADV CAP may help to assess the severity of ADV CAP for the pediatricians to make early intervention.

2. Materials and methods

2.1. Preparation of serum samples

80 children with ADV CAP including 40 with mild ADV CAP (Mild) and 40 with severe ADV CAP (Severe), 10 patients with respiratory syncytial virus (RSV) infection from Guangzhou Women and Children’s Medical Center, were enrolled in this study (flow chart see Supplementary Figure 1). Adenovirus infection was identified by positive multiplex polymerase chain reaction (PCR) for ADVs from nasopharyngeal swabs, sputum, and/ or bronchial alveolar lavage fluid. SP infection was defined by a positive result in bronchoalveolar lavage fluid (BALF) or sputum culture. The diagnosis of severe ADV CAP was defined on the British Thoracic Society guidelines [6]. 40 age-and-sex-matched samples were obtained from healthy children (Control). All blood samples were centrifuged at 1,000 g for 10 min. The serum samples were collected and stored at −80 °C. This study was conducted in compliance with the guidelines of the Declaration of Helsinki (7th revision) after receiving ethical review approval from the Ethics Committee of Guangzhou Women and Children’s Medical Centre, Guangzhou Medical University (NO: IP-2019-28301).

2.2. Protein preparation and iTRAQ labeling

Equal amounts of 5 samples from one group were pooled as one serum sample. According to manufacturer instructions, three pooled samples (Mild, Severe, Control) were treated with a ProteoPrep Blue Albumin and immunoglobulin G (IgG) Depletion kit (Sigma Aldrich, St. Louis, MO, USA) to remove albumin and IgG and protein concentration was measured by Protein Assay kit (Sangon Biotech Co., Ltd. Shanghai, China). 100 μg total protein per sample which was alkylated and subjected to tryptic hydrolysis was reduced. According to iTRAQ Reagents (AB Sciex, Framingham, MA, USA) manufacturer's protocol, every sample was labeled with the iTRAQ tags as follows: Mild group (116 tags), Severe group (118 tags), and Control group (114 tags). All the labeled peptides were pooled together and evaporated to dryness in a vacuum centrifuge, and then analyzed by iTRAQ-based liquid chromatography-mass spectrometry (LC-MS/MS).

2.3. High-pH reversed-phase chromatography

With H2O buffer (NH4H2O, pH = 10), the diluted iTRAQ labeled samples in a final volume of 100 μL were separated by high-performance liquid chromatography (HPLC) on Gemini-NX 3u C18 110A; 150 × 2.00 mm Phenomenex and Gemini 3u C6-Phenyl 110A; 100 × 2.0 mm columns (Phenomenex, Torrance, CA, USA). The separation solvent for reversed phase column consists of H2O (mobile phase A) and 80% acetonitrile (ACN) (mobile phase B) at the flow rate of 0.2 mL/min. The following gradient system was used: 5%–10% B (0–15 min); 15%–28% B (15–48 min); 25%–37% B (48–60 min); 37%–95% B (60–65 min) and 95% B (65–70 min). The elution was monitored by the absorbance at 214/280 nm, and fractions were collected every 50s. All these fractions were pooled and dried in a vacuum centrifuge.

2.4. Reversed-phase liquid chromatography-mass spectrometry (RPLC-MS) analysis

The separation solvent consists of 80% ACN,0.1% formic acid (FA) (mobile phase A), and 0.1% FA (mobile phase B). Peptides were separated by a linear gradient from 5% to 40% of mobile phase B at a flow rate of 300 nl/min for 99 min followed with MS analysis on a Q Exactive system (Thermo Fisher Scientific, Waltham, MA, USA) across the mass range of 350–1,800 m/z in high-resolution mode (m/z 35,000). 100 ms was accumulated per spectrum and a maximum of 20 precursors in every cycle were chosen for fragmentation of every MS spectrum with 120 ms minimum accumulation time for every precursor and dynamic exclusion for 10 s.

2.5. Data analysis

Raw data which was generated from the iTRAQ system was converted into the peak list at the protein level and the peptides data was analyzed by Protein Pilot Software 5.0 utilizing the Paragon protein database search algorithm. The results of MS/MS spectra were searched with the international protein index human sequence database (UniProt Human Proteome database. http://www.uniprot.org/proteomes/UP000005640) with the following parameters: cyste alkylation, methyl methanethiosulfonate; digestion, trypsin (up to one missed trypsin cleavage was allowed). The false discovery rate (FDR) analysis was performed using the integrated tools in Protein Pilot (FDR < 0.01) and the differently expressed proteins that were at least 1.5-fold compared with control, were selected.

2.6. Gene Ontology (GO) and pathway enrichment analysis

The GO and Pathway enrichment analysis of DEPs were performed by ClueGO and ReactomeFIViz of Cytoscape. The terms with P < 0.05 were regarded as significantly enriched.

2.6.1. Protein-protein interaction analysis

Protein-protein interaction analysis on the proteins expressed differently in both Mild vs Control and Severe vs Control was performed by String (https://string-db.org/) with high confidence.

2.7. Western blot analysis

Proteins extracted from serum samples of 125 children (35 Control, 35 Mild, 35 Severe, 10 SP, 10 RSV) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After the separated proteins were transferred onto polyvinylidene fluoride membranes (PVDF; Millipore, Billerica, MA, USA) and blocked overnight, the primary antibodies (all from Abcam, Cambridge, United Kingdom) including Protein S100-A9 (S100A9), Protein S100-A8 (S100A8), Leucine aminopeptidase III (LAP3), and Apolipoprotein A-IV (APOA4) were added for 1 h. Following washing with phosphate buffered saline (PBS), a secondary horseradish peroxidase (HRP)-conjugated antibody was applied and the specific bands on the membranes were visualized using the SuperSignal chemiluminescence system (Promega Corporation, WI, USA). The band intensities were quantified using Image J software (Version 2.0) and normalized to GAPDH. Among them, 75 samples (25 Control, 25 Mild and 25 Severe) were used for proteomics validation. Subsequently, 50 samples (10 Control, 10 Mild, 10 Severe, 10 SP and 10 RSV) were used for biomarkers as distinguish patients with severe ADV CAP from bacterial CAP (SP infection) or viral CAP (RSV infection).
2.8. Statistical analysis

We used McNemars test to calculate P values for paired proportions, and used the Wilcoxon rank sum test for P values of paired numerical comparison. Statistical significance was assessed using analysis of variance and P < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curves were constructed to determine the specificity and sensitivity of individual proteins as potential biomarkers. The area under the ROC curve (AUC) was used as an accuracy index to evaluate the diagnostic performance of the selected protein. MedCalc software was used to perform the ROC analysis.

3. Results

3.1. Characteristics of ADV CAP patients

All children with ADV CAP enrolled in this study were diagnosed by clinical symptoms and lung signs, which was further confirmed by radiology. All patients tested positive for ADV IgM antibody in sera and/or ADV DNA in throat swabs or (and) BALF. Clinical characteristics of all cases are summarized in Table 1. The patients with severe ADV CAP suffered from longer fever and had significantly higher high-sensitivity C-reactive protein (hs-CRP) and LDH levels compared with the mild ADV CAP group (P < 0.05). Chest High-resolution computed tomography (HRCT) was abnormal with more severe multifocal or segmental consolidation with pleural effusion in severe ADV CAP (Figure 1).

3.2. Differently expressed proteins in ADV CAP patients

iTRAQ-based LC-MS/MS was performed in healthy, mild ADV CAP, and severe ADV CAP children. There were 119 DEPs in mild ADV CAP compared with healthy children. 148 DEPs in severe ADV CAP were identified when compared with healthy children (Supplementary Tables 1 and 2). Among these DEPs, 84 proteins were differentially expressed in both mild and severe ADV CAP.

3.3. GO and pathway enrichment analysis

The biological process of GO enrichment analysis for DEPs of mild ADV CAP compared with healthy children were immunoglobulin production, the immune response-regulating cell surface receptor signaling pathway, regulation of lipid localization, protein activation cascade, antimicrobial humoral response and others (Figure 2A). The enrichment analysis for DEPs of severe ADV CAP compared with healthy children were production of molecular mediator of immune response, regulation of response to external stimulus, positive regulation of cytokine secretion, protein-containing complex remodeling, protein activation cascade and others (Figure 2B).

The pathway enrichment analysis was shown in Figure 3. The DEPs of mild ADV CAP compared with healthy children were enriched in *Staphylococcus aureus* infection, cholesterol metabolism, ECM-receptor interaction, IL-17 signaling pathway, and platelet activation (Figure 3A). DEPs of severe ADV CAP compared with healthy children were enriched in *Staphylococcus aureus* infection, IL-17 signaling pathway, cholesterol metabolism, focal adhesion, systemic lupus erythematosus, and the complement and coagulation cascades (Figure 3B).

3.4. The network of differently expressed proteins in both mild and severe ADV CAP

Protein-protein interaction network were constructed on the DEPs both in patients with mild and severe ADV CAP compared to healthy children by STRING. Two main networks were showed that the nodes with highest connectivity were APOH, APOA4, VWF, FGB in one network, and the nodes with highest connectivity were GAPDH, HSP90B1, UBC in another network (Figure 4A and B).

3.5. Proteomics validation and candidate biomarker confirmation of severe ADV CAP patients

To further validate the proteomics results, western blotting was performed to examine the expression levels of 4 proteins (S100A8, S100A9, LAP3, and APOA4) from all 30 individuals (10 Mild, 10 Severe, and 10 Controls). We found that the expression of S100A8, S100A9, LAP3, and APOA4 were significantly upregulated in mild and severe ADV CAP compared to the control group (p < 0.05), among which the LAP3 was the most significantly upregulated protein in severe ADV CAP compared to mild group. (p = 0.0062). These results were consistent with the proteomics analysis by the iTRAQ (Figure 5).

To further confirm that the protein level of LAP3 in serum samples can be used to distinguish patients with severe ADV CAP from bacterial CAP or viral CAP infection, western blotting was performed to detect the expression levels of 3 proteins (S100A8, S100A9, LAP3) from 50 individuals (10 Control, 10 Mild, 10 Severe, 10 SP and 10 RSV). Results showed that S100A8 and S100A9 were highly expressed in children with severe ADV CAP, SP and RSV and the expression of LAP3 was most

### Table 1. Clinical features of 80 patients with human adenovirus community-acquired pneumonia.

| Variable                  | Control (n = 40) | Mild cases (n = 40) | Severe cases (n = 40) | P value* (Mild cases vs Severe cases) |
|---------------------------|-----------------|--------------------|----------------------|--------------------------------------|
| Demographic              |                 |                    |                      |                                      |
| Age (months), median (range) | 45 (12–144)   | 42 (11–113)        | 43 (12–125)          | 0.7835                               |
| Male gender, n (%)       | 19 (47.5)       | 24 (60.0)          | 19 (47.5)            | 0.7800                               |
| Symptoms                 |                 |                    |                      |                                      |
| Fever >7 days, n (%)     | 15 (37.5)       | 19 (47.5)          | 25 (62.5)            | 0.1912                               |
| Cough >7 days, n (%)     | 8 (20.0)        | 11 (27.5)          | 11 (27.5)            | 0.5423                               |
| Shortness of breath, n (%) | 0 (0)          | 11 (27.5)          | 11 (27.5)            | 0.0020                               |
| Laboratory findings     |                 |                    |                      |                                      |
| Abnormal WBC <5.0 × 10^9/L or >12.0 × 10^9/L, n (%) | 20 (50.0) | 30 (75.0) | 30 (75.0) | 0.0531                               |
| Lactate dehydrogenase >500 U/L, n (%) | 8 (20.0) | 30 (75.0) | 30 (75.0) | 0.0001                               |
| C-reactive protein >10 mg/L, n (%) | 11 (27.5) | 27 (67.5) | 27 (67.5) | 0.0021                               |
| Radiology                |                 |                    |                      |                                      |
| Consolidation, n (%)     | 7 (17.5)        | 31 (77.5)          |                      | 0.0001                               |
| Pleural effusion, n (%)  | 0 (0)           | 15 (37.5)          |                      | 0.0002                               |

Data collected from the first laboratory test for the patients on admission;*: p values are two-sided and were adjusted by the Bonferroni method for multiple comparisons testing.
significantly upregulated in severe ADV CAP compared with the other four groups (p < 0.05) (Figure 6).

3.6. Evaluation of LAP3 as a potential diagnostic marker for severe ADV CAP

We next measured the levels of LAP3 in serum samples, established ROC curves to analyze the difference between patients with severe ADV CAP and mild ADV CAP or the control group. Comparing the mild ADV CAP with control groups, the ROC curve areas for LAP3 were found to be 0.849 in patients with mild ADV CAP (95% Confidence Interval: 0.700 to 0.942). The specificity and the sensitivity of LAP3 protein were 81% and 85%, respectively (Figure 7A). In addition, we compared the serum levels of LAP3 protein between severe ADV CAP and control groups. The ROC curve areas of LAP3 were 0.947 (95% Confidence Interval: 0.827 to 0.992). The specificity and the sensitivity for the protein were 95.0% and 90.0%, respectively (Figure 7B). The comparison of severe ADV CAP with mild ADV CAP indicated that LAP3 levels is a useful marker for distinguishing patients with severe ADV CAP from those with mild ADV CAP because the ROC curve area of LAP3 was 0.822 (95% Confidence Interval: 0.669 to 0.925) and the specificity and the sensitivity were 81% and 90.0%, respectively (Figure 7C).

4. Discussion

ADV infection accounts for about 4–10% of CAP in children [10]. ADV causes significant morbidity and mortality, with case fatality rates as high as 12% [16]. Since various acute phase reactants, e.g. CRP, PCT, lactate dehydrogenase could be used as indicators for assessment the severity of CAP, but these indicators are not very specific for a certain pathogen infection [12, 13]. It is necessary to explore novel diagnosis biomarkers. In this study, the serum samples with ADV CAP were collected and differently expressed proteins (DEPs) were detected by iTRAQ-based mass spectrometry. Our data showed that many different expression proteins from both mild and severe ADV CAP compared with healthy children. These DEPs were related to a variety of biological processes and signaling pathways, as identified by Gene Ontology (GO).
and pathway enrichment analysis. The highest connectivity proteins were APOH, APOA4, VWF, FGB and GAPDH, HSP90B1, UBC.

Among the highest connectivity proteins, the expression of the four proteins (S100A8, S100A9 APOA4, and LAP3) were validated, and results indicated that S100A8, S100A9 APOA4 were not significant differences between mild and severe ADV CAP. The function of S100A8, S100A9, APOA4 and LAP3 in ADV CAP is unknown and further investigation is needed. Interestingly, the expression of LAP3 protein was significantly different between mild and severe ADV CAP, which was significantly upregulated in the severe ADV CAP compared to mild ADV CAP. We also analyzed the expression levels of LAP3 in bacterial CAP and viral CAP infection, and found that LAP3 was only highly expressed in children with severe ADV CAP, which was significantly different from the other four groups. Collectively, our results indicated that the LAP3 protein might function as a potential biomarker for severe ADV CAP.

LAP3 is an important member of the LAP family (leucine amino peptidases), which is involved in many pathological disorders including regulating cell proliferation, invasion, and/or angiogenesis of tumors [17]. In addition, LAP is essential for other physiology and biological functions, such as the breakdown of other components involved in

Figure 3. Pathway enrichment analysis for DEPs of mild vs healthy children (A) and severe vs healthy children (B). The size of circle represents P-value. The smaller circle, the lower P-value.

Figure 4. The interaction network of showed DEPs in mild vs healthy children (A) and severe vs healthy children (B).
Figure 5. The validation of four protein by Western blot.

Figure 6. Further confirmation of LAP3 protein as a biomarker for severe ADV CAP by Western blot.
immune responses, or in the breakdown of viral peptides during infections [18]. In our study, GO enrichment and pathway enrichment analysis showed that differently expressed proteins were related to a variety of biological processes and signaling pathways, including immune regulation pathways. In our study, LAP3 was significantly over-expressed in severe ADV CAP compared to mild ADV CAP and healthy children, indicating that LAP3 might be induced by adenovirus severe infection. Consistent with our results, another study reported that LAP was significantly increased in patients with active generalized lymphadenitis (viral infections, toxoplasmosis) [19]. It was previously reported that expression of LAP is inducible by both type I and type II interferons via up-regulation of most components in the similar pathway, indicating that up-regulated LAP expression might play a particularly important role during inflammation induced by ADV infection [20].

4.1. Limitation

Certain limitations of our study should be considered. First, the ability of ADV to cause severe disease may relate to the serotypes of ADVs such as type 7 (HAdV-7), but serotyping was not performed in our present study. Second, a larger scale of study is necessary to validate the diagnostic capability of these proteins before clinical application.

5. Conclusion

The expression of LAP3 in serum samples of patients with severe ADV CAP could be rapidly detected by western blotting. Our present study revealed that LAP3 protein may serve as a candidate biomarker for severe ADV CAP.

Declarations

Author contribution statement

Tingting Shi: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Jun Bai; Li Huang; Dong-Wei Zhang: Analyzed and interpreted the data.
Diyuan Yang: Performed the experiments.
Hui-Feng Fan: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Tongzheng Liu: Contributed reagents, materials, analysis tools or data.
Gen Lu: Conceived and designed the experiments.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest.

Figure 7. Receiver operating characteristic (ROC) curve analysis of LAP3 protein was performed to discriminate Mild from the Control (A), Severe from the Control (B), and Severe from Mild (C).
**Additional information**

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