Research Paper

Identification of nine proteins in whole lung extract derived from bleomycin-induced interstitial pneumonia model mouse by two-dimensional electrophoresis/*in-gel* digestion/ MALDI-TOF MSMS analysis

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Abstract The etiology and pathogenesis of interstitial pneumonia have not been fully elucidated. A proteomics-based approach using two-dimensional electrophoresis/*in-gel* digestion/matrix-assisted laser desorption time-of flight mass spectrometry (MALDI-TOF MS) was applied to identify new candidate biomarkers in whole lung extract from the bleomycin-induced interstitial pneumonia model mouse. First, the mouse lung extract was subjected to two-dimensional electrophoresis and then the bleomycin-induced interstitial pneumonia (BLM-IP) specific spots underwent *in-gel* digestion. The proteins in the specific spots were identified by MALDI-TOF MS and MSMS analyses. Nine proteins including anti-oxidative stress proteins, transgelin-2 (TGLN-2), vimentin, transthyretin, fatty acid-binding protein, and sepiapterin reductase were identified by MALDI-TOF MS and MSMS. The expression levels of these 9 proteins were clearly up-regulated when compared with those of the healthy mouse lung.

In conclusion, the proteomics-based approach enabled us to identify nine up-regulated proteins, especially TGLN-2, in the BLM-IP model mouse. The up-regulated modified TGLN-2 might be an early marker of smooth muscle cells differentiation induced thicken of the alveolar walls and play an important role in pulmonary fibrogenesis.

Key words: bleomycin-induced interstitial pneumonia, whole lung extract, proteomics-based approach, oxidative stress, transgelin-2

Introduction

Idiopathic interstitial pneumonia (IIP) is an intractable disease in which inflammation occurs in the lung interstitium, the walls of alveoli become thick, hard, and show fibrosis, and finally it becomes difficult for oxygen /carbon dioxide exchange to take place in lung alveoli[1)]. The etiology and pathophysiology are still not well-understood, but abnormal wounds are noted due to pathological findings of fibroblast proliferation, extracellular matrix deposition, and scarring[1−3)]. It is considered that excessive fibrosis due to healing and some inflammatory reactions are involved. It is also thought that alveolar epithelial cells acquire the characteristics of mesenchymal cells due to damage to alveolar epithelial cells and cause activation of fibroblasts[4)]. IIP is a life-threatening pathological condition that causes respiratory failure when it progresses. The collective term of inter-

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stitial lung disease comprises various lung conditions, which are characterized by thickening of the alveolar walls by inflammation or fibrosis. Lung inflammation is treated with steroids and immunosuppressants, and pulmonary fibrosis is treated with anti-fibrosis agents such as pirfenidone and nintedanib. However, many cases are treatment-resistant and the outcome is poor in all disease types, which is problematic. The disease is associated with a very poor prognosis, with most patients dying within 5 years of diagnosis.

On developing IP, KL-6, a sialylated carbohydrate antigen, is excessively produced by type II alveolar epithelial cells with inflammation[5]. The extremely high levels of KL-6 in blood showed at the cases of interstitial pneumonia as same as other diseases, such as tuberculosis and malignant tumors, but other lung diseases and bacterial / viral pneumonia were limitedly[6,7]. So, it is not possible to conclude that the levels of KL-6 in blood could be used to diagnose IP. It is important to identify a specific biomarker for differentiations between IP and other diseases.

Bleomycin (BLM) is the most widely used agent to induce pulmonary fibrosis in experimental animals. After the systemic delivery of BLM, endothelial cells were directly injured, and this was subsequently followed by epithelial cell injury, inflammation, and fibrosis[8]. With the intratracheal, intranasal, or vascular administration of BLM, lung epithelial cells were initially injured, but the development of endothelial cell damage has not been fully elucidated. It would be a very important to identify new biomarker for the understanding critical cause of pulmonary fibrogenesis and thickening of the alveolar walls in the BLM-induced interstitial pneumonia (BLM-IP).

Proteomics-based identification facilitates high-throughput studies of protein expression, and enables the identification of molecular mechanisms that are responsible for the development of specific diseases. For example, in comparison with RNA-sequence or microarray-based profiling of gene expression, which were previously applied to idiopathic pulmonary fibrosis, proteomics analysis offers various advantages[9,10]. In particularly, it indicates the effective existence of functional proteins in clinical samples. In addition, a high transcription level leading to an abundant quantity of messenger RNA does not always mean an abundance of the corresponding protein or its effectual activity. Therefore, the proteomics-based technique has significant strategic benefits for the identification of specific diagnostic biomarkers and new therapeutic targets. Previously, our group reported that new biomarkers, 4 citrullinated proteins could be used to differentiate between rheumatoid arthritis and osteoarthritis patients by the proteomics-based technique[11].

In the present study, we applied the novel proteomics-based investigation using two-dimensional electrophoresis/in-gel digestion/matrix-assisted laser desorption time-of flight mass spectrometry (MALDI-TOF MS) to investigate new biomarkers in whole lung extract from the BLM-IP model mouse, and identified nine up-regulated anti-oxidative and vesicle stress-related proteins.

Materials and Methods

Culture of mice for drug-induced pulmonary fibrosis

The Institutional Animal Care and Use Committee of Osaka Medical and Pharmaceutical University approved all of the following research protocols (approval ID: 27053), including the surgical procedures and animal care, and all methods were performed in accordance with the relevant guidelines and regulations.

Female 13−14-week-old C57BL/6J mice (SHIMIZU Laboratory Supplies, Kyoto, Japan) were anesthetized with an i.p. injection of 400 mg/kg 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich Japan K.K.). The administered BLM was prepared by mixing sterile BLM sulfate powder (Nippon Kayaku, Tokyo, Japan) with normal sterile saline. A dose of 3 mg of BLM in a total volume of 100 μL of sterile saline was injected subcutaneously by osmotic minipump (Alzet 2010, DURECT, Cupertino, CA, USA) from days 0 to 7[12,13]. After anesthetizing the model mouse, the thorax was opened and the cardiopulmonary was exposed, we were injected from the right ventricle for pulmonary perfusion using 2 mL of saline containing 100 U of heparin. The mice were sacrificed at 28 days after the initiation of bleomycin injection, the lungs were harvested for histological analysis, and stored at −80 degrees prior to use.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The lungs were homogenized by ProteoExtract™ Complete Mammalian Proteome Extraction Kit (Merck Millipore, Darmstadt, Germany) and cOmplete Mini™ (Roche Diagnostics, Indianapolis, IN, USA). Homogenates were mixed with lysis buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3−10, and 1% dithiothreitol). Protein concentrations of these samples were measured by Protein

Assay System™ (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots containing 100 micrograms of proteins were applied overnight to Immobiline Drystrip™ (GE Healthcare Bio-Science, Piscataway, NJ, USA) by in-gel rehydration. The rehydrated gels were then gently dried with filter paper to remove excess fluid and isoelectric focusing (IEF) was performed in a Pharmacia Hoefer Multiphor II™ electrophoresis chamber (GE Healthcare Bio-Science, Piscataway, NJ, USA), according to the manufacturer's instructions. Two-dimensional SDS-PAGE was performed in 9−18% acrylamide gradient gels using an Iso-Dalt electrophoresis chamber. The 2-D gels were stained with SYPRO Ruby (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The SYPRO Ruby-stained protein spots were detected using Molecular Imager FX™ (Bio-Rad Laboratories, Hercules, CA, USA) and were subjected to *in-gel* digestion. Image analysis and database management were done using Image Master Platinum image analysis software™ (GE Healthcare Bio-Science, Piscataway, NJ, USA).

In-gel digestion and mass spectrometric identification of proteins

This work was performed essentially as described elsewhere[4]. Protein spots were excised from the dried silver stained 2-D gels, and rehydrated for 20 min in 100 mM NH_4HCO_3. The gel spots were then destained for 20 min in a solution of 15 mM potassium ferricyanide and 50 mM thiosulfate, rinsed twice in Milli-Q water, and finally dehydrated in 100% acetonitrile until they turned opaque white. The gel pieces were then dried in a vacuum centrifuge, and subsequently rehydrated in a digestion solution consisting of 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 0.1 μg/μL modified sequence-grade trypsin (Promega). After overnight incubation at 37 degrees, the digestion was terminated in 5% trifluoroacetic acid (TFA) for 20 min. Peptides were extracted 3 times (20 min each) with 5% TFA in 50% acetonitrile, and the extracted peptides were pooled and dried in a vacuum centrifuge. The peptides were purified with ZipTipC18™ (Millipore) following the manufacturer's protocol and analyzed by Ultraflex TOF/TOF (Bruker Daltonics) MALDI mass spectrometer and MASCOT database software (Matrix Science).

Results and Discussion

In the present study, we applied a novel proteomics-based investigation to identify new candidate biomarkers in the BLM-IP model for the prediction of etiologies of lung pulmonary fibrosis in comparison with a normal mouse.

As shown in Fig. 1, microscopy images of Masson trichrome- stained lung tissues show a definite change between the BLM-IP model mouse and normal mouse. A lung section from the BLM-IP model mouse showed the diffuse increase collagen deposition compared with normal lung. As expected, we observed lung fibrosis with collagen deposition in lung interstitium 28 days after BLM administration.

As shown in Fig. 2, the SYPRO Ruby-stained protein patterns of the BLM-IP model mouse and normal mouse overlapped. Seventeen spots were different between the

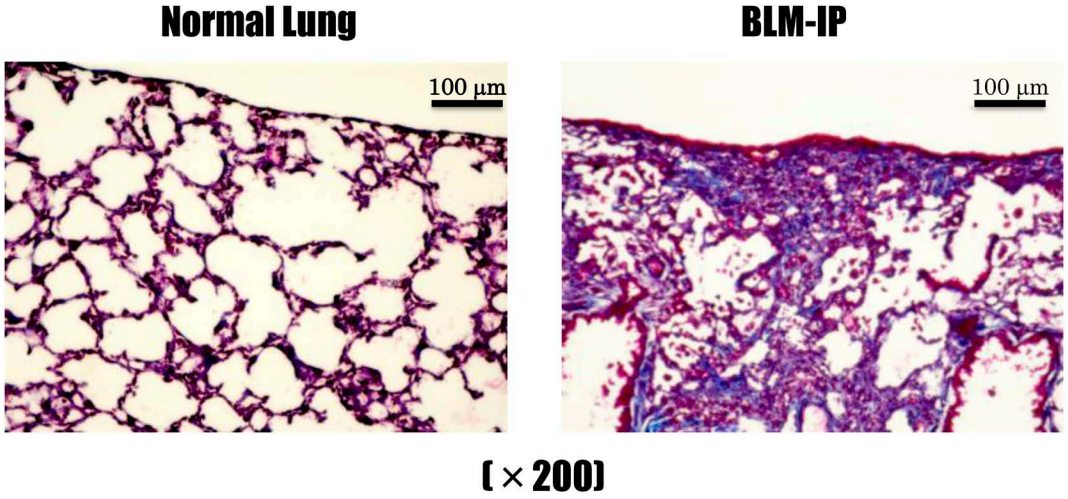

Fig. 1. Representative microscope images of Masson trichrome-stained lung tissue sections of normal and BLM-IP model mice 28 days after treatment.
The scale bar indicates 100 μm.

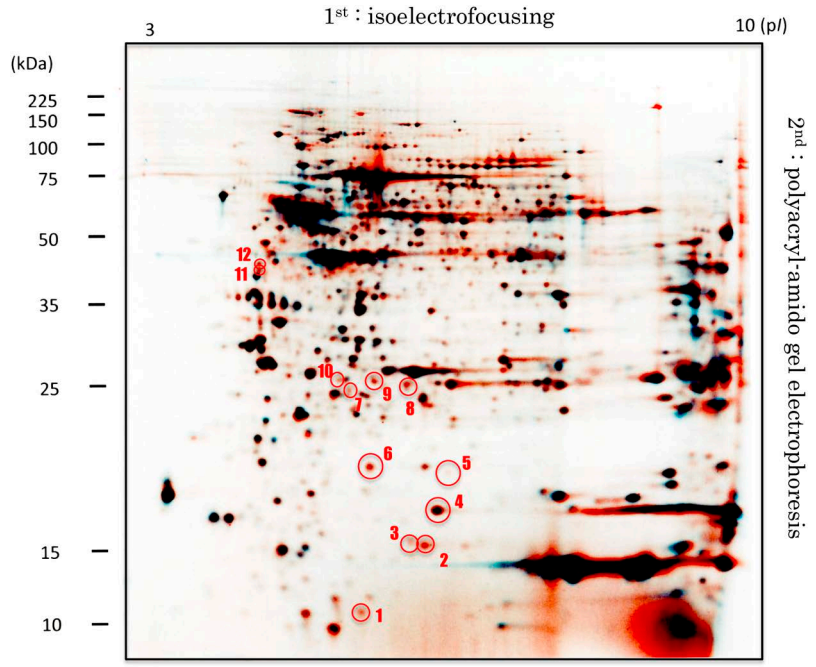

Fig. 2. An overlaid image of the two-dimensional gel electrophoresis of proteins in whole lung extract derived from BLM-IP model mouse (red) and reference healthy mouse (green).
The red numbers indicate up-regulated proteins in the BIM-IP model mouse compared with the reference mouse.

BLM-IP model mouse and normal mouse. Twelve of these 17 spots showed higher expressions in the BLM-IP mouse than those of the normal mouse, and five spots showed lower expression in the BLM-IP group than in the normal mouse. As the results of MALDI-TOF-MSMS analysis coupled with the MASCOT database search, the 12 up-regulated proteins in the BLM-IP group could be identified, but a single spot could not be identified, as shown in Table 1. They included two anti-oxidative stress proteins (spot Nos. 4 and 8), a thyroid hormone-binding protein (No. 2), an intracellular carrier for active lipids (No. 3), actin-related proteins (Nos. 5 and 6), a molecule involved in cholesterol metabolism (No. 7), a molecular chaperon (No. 9), a molecule involved in tetra-hydrobiopterin biosynthesis (No. 10), and cytoskeleton proteins (Nos. 11 and 12). Interestingly, spot Nos. 5 and 6 were also identified as transgelin-2 (TGLN-2), but the isoelectric point (pI) of spot No. 6 was clearly lower than that of spot No. 5. It is of interested whether this modified TGLN-2 molecule leads to accelerated lung fibrosis compared with the native TGLN-2. So, we speculated that this lower shift of pI may be caused by a post-translational modification, such as phosphorylation, but this MSMS analysis unfortunately could not clarify any evidence of the modifications. And so, we further examined to identify a post-translational modification of the TGLN-2 molecule by the electrophoresis/western-blot/ ECL detection. As the result, the phosphorylation could not be confirmed, but it might be still caused by a *S*-sulfonation or *S*-nitrosylation in which pI shifts to the acidic side. On the other hand, spot Nos. 10 and 11, both identified as vimentin (VIM), had the same pI, but the molecular weight of spot No. 11 was definitely higher than that of spot No. 10, possibly due to modification, such as citrullination, glycosylation, or ubiquitination.

Fig. 3a)−h) show MSMS spectra of the tryptic *in-gel* digestions of spots (Nos. 2, 3, 4, 7, 8, 9, 10, 11, and 12) in the whole lung extract of the BIM-IP model mouse. The MSMS spectra obtained from spot No. 2 matched the sequence of two peptides derived from transthyretin (TTR). Figure 3a shows the MSMS spectra from the sequence of a tryptic peptide from the 84th to 90th TTR. In the same way, other tryptic peptides were also identified by MSMS analysis coupled with the MASCOT database search. The MSMS spectra matched the sequences from tryptic peptide of fatty acid-binding protein 5 (FABP5), copper-zinc superoxide dismutase (SOD-1), Apolipoprotein A-1 (Apo A-1), peroxiredoxin-6 (Perx-6), heat shock protein beta-1 (HSP *b*-1), sepiapterin reductase (SPR), and VIM, respectively.

Kulkarni et al.[14] reported a novel proteomics approach to clarify the profiles of expressed proteins in cultured fibro-

Table 1. Up-regulated proteins from 2D gel spots derived from whole lung extract of the bleomycin-induced interstitial pneumonia model mouse by MALDI-TOF/TOF and MASCOT database

Spot No.	Name	MW in database (KDa)	Observed MW (KDa)	Description	pI	Localization	Mowse Score
1	ND	—	—	—	—	—	—
2	Transthyretin	16	15	TTR, thyroid hormone-binding protein	5.77	cytoplasm, secreted	43
3	Fatty Acid-binding Protein	15	15	FABP5, epidermal type	6.14	cytoplasm	51
4	Superoxide Dismutase Cu-Zn	16	16	SOD1, Antioxidant	6.02	cytoplasm,	103
5	Transgelin-2	23	20	TAGLN2, protein binding, epithelial cell differentiation	8.39	cytoskeletal protein	77
6	Transgelin-2	23	20				143
7	Apolipoprotein A-I	30	25	ApoA1, cholesterol transporter	5.64	secreted	61
8	Peroxiredoxin-6	25	25	PRDX6, antioxidant	5.71	cytoplasm	198
9	Heat shock protein beta-1	23	25	HSPB1, estrogen-regulated	6.12	cytoplasm	104
10	Sepiapterin reductase	28	26	SPR, oxidoreductase	5.58	cytoplasm	79
11	Vimentin	53	45	intermediate filaments	5.06	cytoplasm	63
12	Vimentin	53	46				46

ND: not identification.

blasts of mouse lung tissue after the intranasal administration of BLM. The expression levels of HSPs, TGLN-2, and VIM were up-regulated and those of MMP-9 were down-regulated compared with the normal mouse. They suggested that the BLM-induced up-regulated proteins were closely associated with phosphoinositide-3-kinase/protein kinase B (PI3K/Akt), and that nitric oxide signaling pathways are important for the regulation of pulmonary fibrosis[15,16]. On the other hand, Della et al.[17] reported the expression levels in a model mouse of lung fibrosis induced by the intratracheal instillation of a single dose of BLM. The levels of Perx-6, TGLN-2, VIM, and HSP all showed down-regulated patterns, with all results being the opposite of our MS analysis. We considered that this discrepancy was likely due to the route of administration of BLM, and so the expression pattern of cytokines, proteases/anti-proteases, growth factors, and signaling pathways involved in pulmonary fibrosis were different. Chua F et al.[19] also reported that a single intratracheal instillation of BLM rapidly leads to severe bronchiocentric fibrosis, while intravenous or intraperitoneal routes of administration characteristically induce subpleural scarring, and mild intermittent fibrosis can result from repeated BLM dosing similar to the induction of human pulmonary fibrosis.

In the present study, we identified 2 kinds of up-regulated anti-oxidative proteins in the BIM-IP model mouse (SOD-1 and Perx-6). Oxidative stress caused by reactive oxygen species is involved in the pathophysiology of IIP, and oxidative stress is associated with damage to lung epithelial cells and vascular endothelial cells and abnormal tissue repair of the lung, and thus induces fibrosis[20]. Schamberger AC et al.[21] reported that anti-oxidative molecules, such as SOD, glutathione-3-peroxidase, and HSP b-1, are highly expressed in interstitial lung diseases of humans and model mice, as in the present study. HSP *b*-1, also known as HSP27, is a small heat shock protein involved in many cellular processes and protects cells against oxidative stress and controls cell apoptosis.

As shown in Figs. 4 and 5, the MSMS spectra obtained from spot Nos. 5 and 6 matched the sequences of two and six tryptic peptides of the same protein, TGLN-2, in the lung tissue extract of the BIM-IP model mouse. TGLN-2, which belongs to the actin-binding protein family, is an early marker of smooth muscle cell differentiation. The basic function of TGLN-2 is to regulate the actin cytoskeleton through actin binding, and eventually participate in processes involving cytoskeleton remodeling. As the present results, we considered that the differentiation of smooth muscle cells and reconstruction of the cytoskeleton would

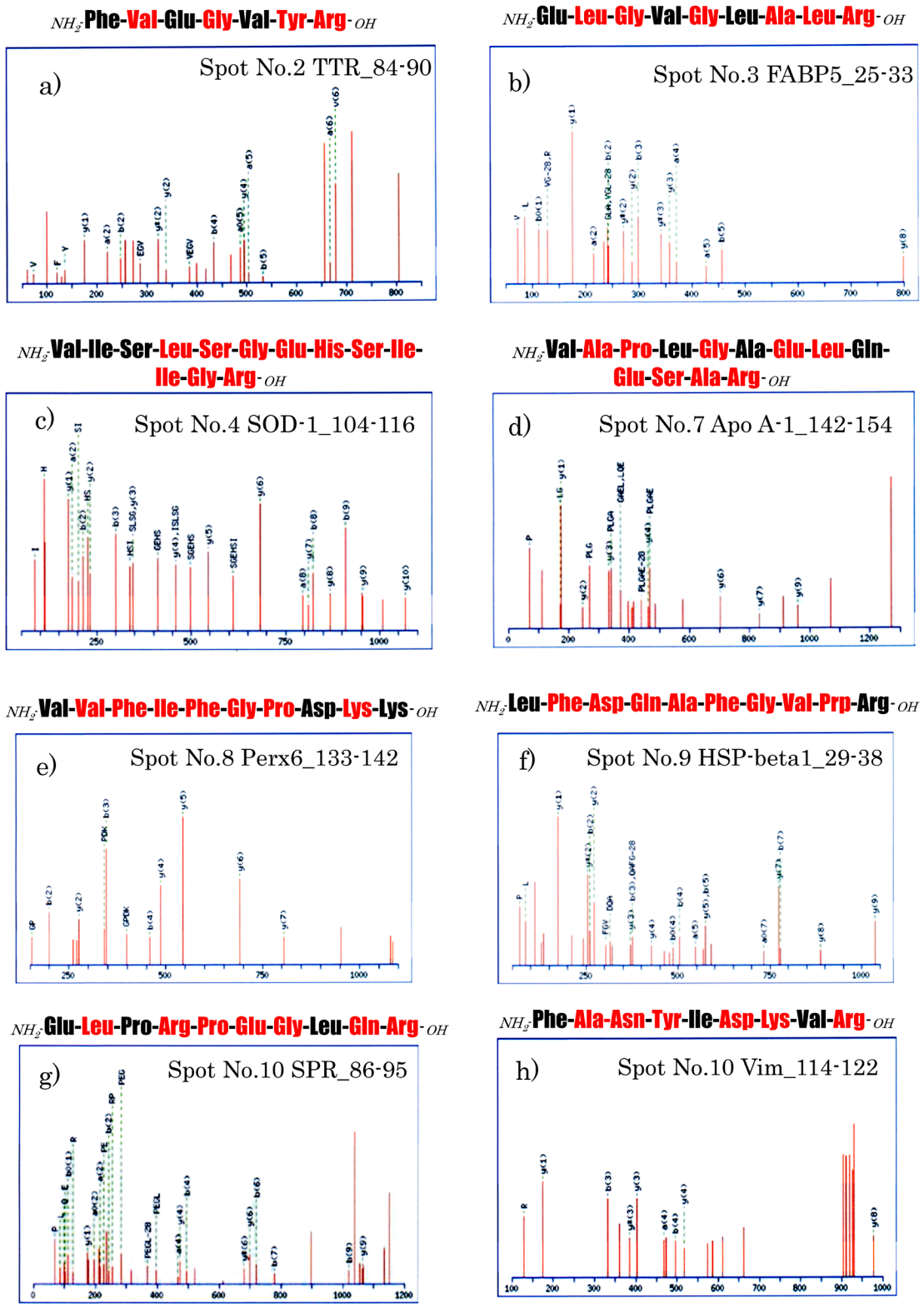

Fig. 3. The MSMS spectra of the tryptic *in-gel* digestions of spots (Nos. 2, 3, 4, 7, 8, 9, 10, 11, and 12) in whole lung extract of the BIM-IP model mouse. All Mowse scores of MSMS ion spectra indicate identity or extensive homology ($p<0.05$), which strongly supported these identifications.

a) MALDI-TOF/TOF mass spectra of peptides from the *in-gel* digest of 2D gel spot No. 2. The red letters indicate the amino acids of the tryptic peptide of TTR. The numbers show the residues of identified sequences of the TTR.

b) FABP-5, c) SOD-1, d) Apo A-I, e) Perx-6, f) HSP *b*-1, g) SPR, and h) Vim also present the MSMS spectra of tryptic peptides; the red letters and numbers are the same as above-mentioned.

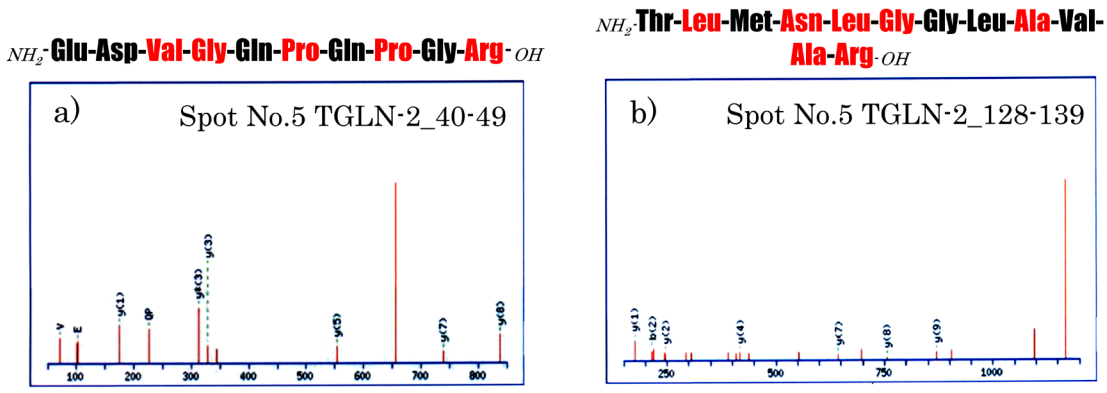

Fig. 4. MALDI-TOF/TOF mass spectra of peptides from the *in-gel* digest of 2D gel spot No. 5.
The red letters indicate the amino acids of the tryptic peptide of TGLN-2. The numbers show the residues of identified sequences of TGLN-2.

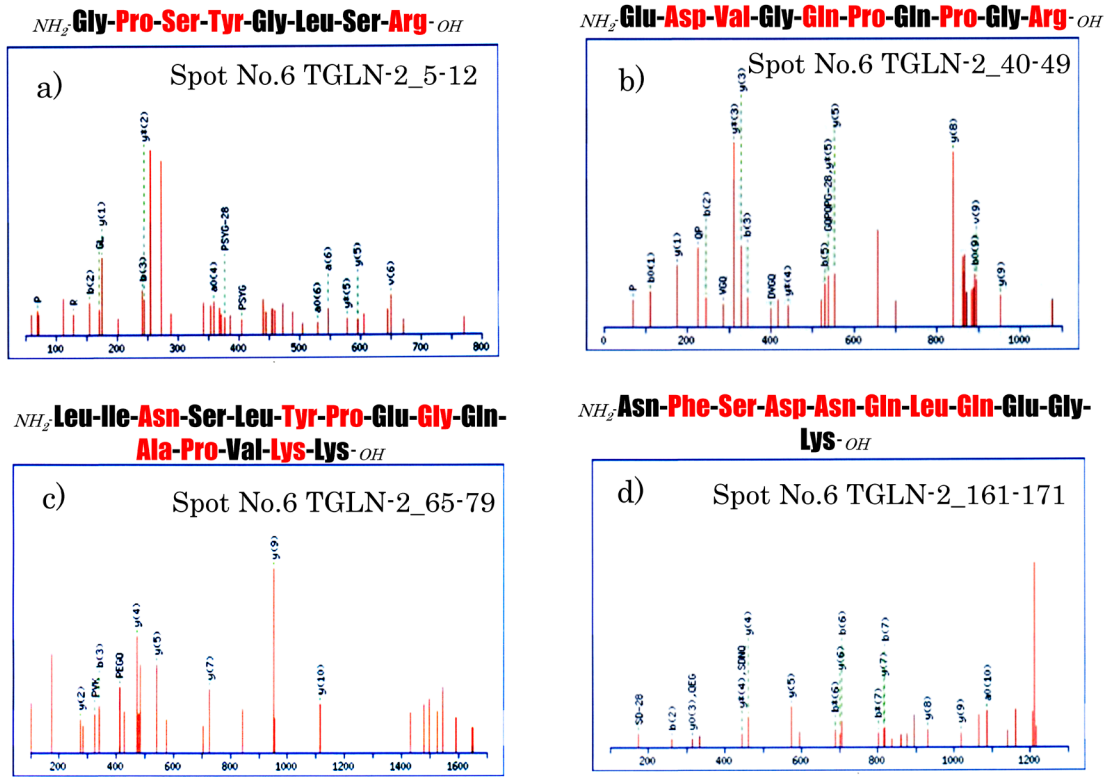

Fig. 5. MALDI-TOF/TOF mass spectra of peptides from the *in-gel* digest of 2D gel spot No. 6.
The red letters indicate the amino acids of the tryptic peptide of TGLN-2. The numbers show the residues of identified sequences of TGLN-2.

also occur in the BLM-IP mouse. Yu H et al.[22] reported that TGLN-2 mRNA expression in isolated alveolar epithelial type II cells was significantly up-regulated after BLM administration compared with the control mouse. This up-regulation was also noted in lung species of IP fibrosis patients. This high expression of TGLN-2 was recognized in fibroblast-to-myofibroblast transdifferentiation, which is a process leading to endothelial-to-smooth muscle cell transdifferentiation, strongly suggesting that TGLN-2 expression coincides with cellular plasticity. They demonstrated that the up-regulated TGLN-2 plays an important role in pulmonary fibrogenesis.

Nonaka et al.[23] reported that a case of tracheal amyloidosis discovered during observation of interstitial pneumonia. Although the authors considered to be a coincidence of tracheal amyloidosis and IP, there are also some reports of the diffuse alveolar septal amyloidosis. They suggested that further study is needed to resolve the relationship between

IP and tracheal localized amyloidosis. Previously, our group had first identified the *S*-sulfonation of transthyretin (TTR) in patient with TTR-related amyloidosis by the novel proteomics-based analysis. The *S*-sulfonation of TTR is an important first step in the formation of TTR-related amyloid fibril. The shift of the pI value in this modification will be similar with that of the phosphorylation, and the m/z shift is the same, +80 Da. At the present study, the levels of total TGLN-2 in the lung extract of BIP model mouse clearly increased than that the reference mouse. We speculated that this modification on the TGLN-2 might be one of the trigger steps to induce lung fibrogenesis in the BIP model mouse as our previous report.

In conclusion, we identified nine proteins in whole lung extract derived from the BLM-IP model mouse by two-dimensional electrophoresis/*in-gel* digestion/MALDI-TOF MSMS analysis. These up-regulated proteins, especially modified TGLN-2, might be an early marker of smooth muscle cells differentiation induced thicken of the alveolar walls and play an important role in pulmonary fibrogenesis due to exposure to BLM.

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Conflict of Interest

The authors declare no conflicts of interest, financial or otherwise.

References

1) Oliveira DS, Araújo Filho JA, Paiva AFL, Ikari ES, Chate RC, et al: Idiopathic interstitial pneumonias: Review of the latest American Thoracic Society/European Respiratory Society classification. *Radial Bras* 51(5): 321−327, 2018.
2) Gross TJ, Hunninghake GW: Idiopathic pulmonary fibrosis. *N Engl J Med* 345: 517−525, 2001.
3) Azad N, Iyer AKV, Wang L, Liu Y, Lu Y, et al: Reactive oxygen species-mediated p38 MAPK regulates carbon nanotube-induced fibrogenic and angiogenic responses. *Nanotoxicology* 7(2): 157−168, 2013..
4) Parimon T, Yao C, Stripp BR, Noble PW, Chen P: Alveolar Epithelial Type II Cells as Drivers of Lung Fibrosis in Idiopathic Pulmonary Fibrosis. *Int J Mol Sci* 21(7): 2269−2290, 2020.
5) Ishikawa N, Hattori N, Yokoyama A, Kohno N: Utility of KL-6/MUC1 in the clinical management of interstitial lung diseases. *Respir Invest* 50: 3−13, 2012.
6) Kohno N, Kyoizumi S, Awaya Y, Fukuhara H, Yamakido M, et al.: New serum indicator of interstitial pneumonitis activity. Sialylated carbohydrate antigen KL-6. *Chest* 96: 68−73,1989.
7) Kohno N, Inoue Y, Hamada H, Fujioka S, Fujino S, et al.: Difference in serodiagnostic values among KL-6-associated mucins classified as cluster 9. *Int J Cancer Supplement* 8: 81−83, 1994.
8) Matsumoto T, Urushido M, Ide H, Ishihara M, Hamada-Oda K, et al: Small heat shock protein beta-1 (HSPB1) is upregulated and regulates autophagy and apoptosis of renal tubular cells in acute kidney injury. *PLoS ONE* 10(50): e0126229, 2015.
9) Tzouvelekis A, Patlakas G, Bourous D: Application of microarray technology in pulmonary diseases. *Respir Res* 5: 26−43, 2004.
10) Kan M, Shumyatcher M, Himes BE: Using omics approaches to understand pulmonary diseases. *Respir Res* 18: 149−168, 2017.
11) Tabushi Y, Nakanishi T, Takeuchi T, Nakajima M, Ueda K, et al: Detection of citrullinated proteins in synovial fluids derived from patients with rheumatoid arthritis by proteomics-based analysis. *Ann Clin Biochem* 45: 413−417, 2008.
12) Harrison JH, Lazo JS: High dose continuous infusion of bleomycin in mics: a new model for drug-induced pulmonary fibrosis. *J Pharmacol Experi Therapeut* 243(3): 1185−1194, 1987.
13) Kotani T, Masutani R, Suzuka T, Oda K, Makino S, et al: Anti-inflammatory and anti-fibrotic effects of intravenous adipose-derived stem cell transplantation in a mouse model of bleomycin-induced interstitial pneumonia. *Sci Rep* 7(1): 14608−14617, 2017.
14) Kulkarni TM, Dutta S, Iyer AKV, Venkatadri R, Kaushik V, et al: A proteomics approach to identifying key protein targets involved in VEGF inhibitor mediated attenuation of bleomycin-induced pulmonary fibrosis. *Proteomics* 16(1): 33−46, 2016.

15) Lu Y, Azad N, Wang L, Lyer AKV, Castranova V, et al: Phosphatidylinositol-3-kinase/akt regulates bleomycin-induced fibroblast proliferation and collagen production. *Am J Respir Cell Mol Biol* 42(4): 432−441, 2010.
16) Lyer AKV, Ramesh V, Castro CA, Kaushik V, Kulkarni YM, et al: Nitric oxide mediates bleomycin-induced angiogenesis and pulmonary fibrosis via regulation of VEGF. *J Cell Biochem* 116(11): 2484−2493, 2015.
17) Della LV, Cecchettini A, Comelli L, Ucciferri N, Di PC, et al: A proteomics approach to the study of bleomycin-induced lung fibrosis. *J Transl Sci* 5: 1−7, 2018.
18) Korfei M, Beck von der D, Henneke I, Markart P, Ruppert C, et al: Comparative proteome analysis of lung tissue from patients with idiopathic pulmonary fibrosis (IPF), non-specific interstitial pneumonia (NSIP) and organ donors. *J Proteomics* 85: 109−128, 2013.
19) Chua F, Gauldie J, Laurent GJ: Pulmonary fibrosis: searching for model answers. *Am J Respir Cell Mol Biol* 33(1): 9−13, 2005.
20) Fois AG, Paliogiannis P, Sotgia S, Mangoni A, Zinellu E, et al: Evaluation of oxidative stress biomarkers in idiopathic pulmonary fibrosis and therapeutic applications: a systematic review. *Respir Res* 19: 51−63, 2018.
21) Schamberger AC, Schiller HB, Fernandez IE, Sterclova M, Heinzelmann K, et al: Glutathione peroxidase 3 localizes to the epithelial lining fluid and the extracellular matrix in interstitial lung disease. *Sci Report* 6: 29952−29966, 2016.
22) Yu H, Konigshoff M, Jayachandran A, Handley D, Seeger W, et al: Transgelin is a direct target of TGF-b/Smad3-dependent epithelial cell migration in lung fibrosis. *FASEB J* 22(6): 1778−1789, 2008.
23) Nonaka Y, Shimazu K, Fukushima Y, Ebihara M: A case of tracheal amyloidosis discovered during observation of interstitial pneumonia. *J Jap Respir Soc* 41(1): 10−13, 2003.