**Original
Article**

Fibulin-5 Protein Is Reduced in the Lung of Patients with Spontaneous Pneumothorax Who Are Under 25 Years Old

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Purpose: To clarify whether fibulins-5 is associated with primary spontaneous pneumothorax (PSP) in young PSP patients.

Methods: Forty-six surgically resected, fresh lung specimens were used. Patients were divided into 3 groups: younger than 25 years with pneumothorax (group Y), 25 years or older with pneumothorax (group O), and without pneumothorax (group C). Chest X-ray, computed tomography data, height/width ratio (H/W) and anteroposterior/transverse diameter ratio (a/b) were measured. Elastica van Gieson staining and immunofluorescence staining for fibulin-5 were performed. Fibulin-5 mRNA expression and protein levels were measured by real-time PCR and western blotting. Direct sequences of the fibulin-5 gene in PSP patients were performed.

Results: The mean H/W ratio in group Y was significantly larger than that in the other groups ($p < 0.01$). The mean a/b ratio in group Y was significantly smaller than that in the other groups ($p = 0.02$). Fibulin-5 mRNA expression was not significantly different among the groups ($p = 0.64$). The relative intensity of fibulin-5 protein in group Y was significantly lower than that in group O ($p = 0.006$), with no significant differences between groups O and C ($p = 0.14$).

Conclusions: We showed that fibulin-5 is reduced in patients with PSP who are younger than 25 years.

Keywords: fibulin-5, pneumothorax, bulla, Western blot, real-time reverse transcriptase polymerase chain reaction

Introduction

A high incidence of pneumothorax is a relatively common problem in Japan. Pneumothorax is defined as the presence of air in the pleural space. From a clinical stand point, spontaneous pneumothorax is classified as primary

or secondary.¹⁾ Primary spontaneous pneumothorax (PSP) is defined as pneumothorax occurring in patients with no underlying pulmonary disease. PSP commonly occurs in young, thin males. PSP has an incidence of 7.4-8 cases (age-adjusted incidence) and 1.2-6 cases/100000 population per year in males and females, respectively.²⁾

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Received: August 8, 2011; Accepted: October 18, 2011

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Secondary spontaneous pneumothorax (SSP) usually occurs in older people with underlying pulmonary disease, such as chronic obstructive lung disease. SSP also occurs in patients with congenital connective tissue disorders such as Marfan syndrome³⁾ and Ehlers-Danlos Syndrome.⁴⁾

PSP is usually caused by rupture of pleural blebs or bullae.⁵⁾ However, the exact pathogenesis of blebs or bullae and the reason why PSP frequently occurs in young, thin males are still unknown. Furthermore, in the clinical situation, many young patients who have no apparent risk factors, such as smoking, suffer from this disease.

The fibulin family is a group of widely expressed extracellular matrix (ECM) proteins localized in the basement membrane, stroma, and ECM fibers.⁶⁾ They mediate cell-to-cell and cell-to-matrix communication and provide organization and stabilization to ECM structures during organogenesis and vasculogenesis.⁷⁾ Fibulin-5 is a recently identified fibulin family.⁸⁾ Fibulin-5 contributes to the formation of elastic fibers by binding to structural components including tropoelastin and fibrillin-1.⁹⁾ Studies in fibulin-5 deficient mice have shown that fibulin-5 is necessary for the biosynthesis of elastic fibers.¹⁰⁾ Fibulin-5 is thought to be an adaptor for binding to microfibrils for the formation of elastic fibers.¹¹⁾

In this study, we hypothesized that PSP in young patients is induced by previously unknown causes, which is different from SSP. We also hypothesized that fibulins-5 is associated with PSP in young PSP patients. We investigated the gene expression, protein, and tissue localization of fibulins-5 in patients with spontaneous pneumothorax patients and controls. Furthermore, we also analyzed clinical data on the shape of the thorax to reveal features of young PSP patients.

Materials and Methods

Patients

Surgically resected fresh lung specimens were obtained from 36 patients with pneumothorax and 6 patients with metastatic colorectal cancer as controls at Saiseikai Wakayama Hospital from June 2009 to September 2010. Four specimens were also obtained as controls when administrative autopsy was performed in our institute. Immediately after surgery, samples were snap-frozen in liquid nitrogen and stored at approximately -80°C . To analyze young PSP patients, patients with pneumothorax were divided into 2 groups. Patients younger than 25 years with pneumothorax were considered as the young

Fig. 1 a: Chest X-ray showing an AP view. The H/W ratio was calculated as height (H) divided by width (W).

b: Chest CT scan. The b/a ratio in the CT image was calculated as the anteroposterior diameter (a) divided by the transverse diameter (b) at the level of the first carina.

group (Y group, $n = 14$), those aged 25 years or older with pneumothorax were considered as the old group (O group, $n = 22$). Patients without pneumothorax were also defined as the control group (C group, $n = 10$). The study was approved by the ethics committee of Saiseikai Wakayama Hospital, and written informed consent was obtained from each patient.

Clinical data analysis for chest deformity

The anteroposterior (AP) view in chest X-ray and computed tomography (CT) scans was used to evaluate chest deformity of patients. The H/W ratio was calculated as height (H) divided by width (W) in the chest X-ray AP view (**Fig. 1a**). The b/a ratio in the CT image was calculated as the anteroposterior diameter (a) divided by the transverse diameter (b) at the level of the first carina (**Fig. 1b**). Height and weight data were also obtained.

Visualization of elastin

Elastin was visualized in paraffin section ($5\ \mu\text{m}$) by elastica van Gieson staining (**Fig. 2a** and **2b**).

Immunofluorescence staining for fibulin-5

Tissue sections were deparaffinized and rehydrated through 100%, 95% and 90% ethanol solutions. For antigen retrieval, the slides were heated in a hot water bath for 15 min at 95°C followed by a 20-min cool-down period at room temperature. The sections were incubated with an anti-fibulin-5 polyclonal antibody (1:500, SC-30170, Santa Cruz, CA) for 60 min at room temperature. FITC-anti-rabbit IgG (Sigma, St. Louis, MI) was used as a secondary antibody (**Fig. 2c** and **2d**).

Fig. 2 Visualization of elastin and co-localization with fibulin-5. Elastica van Gieson staining (upper) and immunofluorescence staining for fibulin-5 (lower) are shown. a and c are the same samples in group Y. b and d are the same samples in group O.

Real-time RT-PCR

After the frozen samples were homogenized, total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription to cDNA was performed using Superscript™ RNase H-Reverse Transcriptase (Invitrogen), and random oligonucleotide primers, synthesized according to the standardized protocol, were provided by the manufacturer.

To quantify the levels of mRNA encoding fibulin-5, the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) was employed. GAPDH was used as an internal standard. All determinations were analyzed in triplicate in TaqMan® Universal Master Mix (Applied Biosystems) on 96-well optical PCR plates. Appropriate primers and probes were purchased from commercially available TaqMan gene expression assays (fibulin-5: Hs00197964_m1, GAPDH: Hs99999905_m1, Applied Biosystems). The intensities of expression of interest were expressed relative to the GAPDH intensities from the same sample.

Western blot analysis

For protein analysis, 100 mg of tissue were homogenized and lysed in 500 µg sample buffer (0.125 ml/l Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 2% mercaptoetha-

nol, and 0.03 mmol/l bromophenol blue). Five micrograms of protein from each sample were fractionated by 10% SDS polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Biorad Laboratories, Richmond, CA). The membrane was blocked with phosphate-buffered saline (PBS), containing 5% nonfat dry milk for 1 h at room temperature, and incubated overnight at 4°C with primary antibodies directed against fibulin-5 (1:1000, SC-30170, Santa Cruz) and β-actin (1:1500, AC15, Sigma). After washing 3 times with PBS, the membrane was incubated in horseradish-peroxidase-conjugated secondary antibodies at room temperature for 1 h. Bands were developed with the ECL plus detection system (Amersham Bioscience, Buckinghamshire, UK) and quantified by Scion image software (Scion Corp., MD). The intensities of bands were expressed relative to the β-actin intensities from the same sample.

Statistical analysis

All the values were calculated as the mean ± standard deviation (minimum–maximum). Results were subjected to statistical analysis using the χ^2 test and one-factor ANOVA. Values of $p < 0.05$ were considered to be significant.

Table 1 Patient characteristics and clinical data in each group

	Y	O	C	p-value (Y vs O)
Patients characteristics				
Number of cases	14	22	10	
Age (years)	19 (16–24)	46 (27–72)	60 (14–79)	<0.01
Sex				
Male	13	22	3	0.78
Female	1	0	7	
Smoking status				
smoker	4	22	3	<0.01
non-smoker	10	0	5	
uncertain	0	0	2	
Clinical data				
Height (m)	1.71	1.72	1.5	0.51
Weight (kg)	56	61.5	57	0.045
H/W ratio	1.19	1	0.99	<0.01
a/b ratio	0.3	0.34	0.4	0.22

Results

Patients' characteristics and clinical data analysis

Patients' characteristics and clinical data of each group are summarized in **Table 1**. Thirty-five of 36 (97%) patients with pneumothorax were males. All patients in group O were smokers, which is in contrast to 4 of 14 (28.6%) smokers in group Y. There was no significant difference in height between group Y and O ($p = 0.51$). The mean weight in group Y was significantly smaller than that in group O ($p = 0.045$). The mean H/W ratio in group Y was significantly larger than group O ($p < 0.01$). There was no significant difference in a/b ratio between group Y and O ($p = 0.22$).

Localization of fibulin-5

Elastica van Gieson and immunofluorescence staining for fibulin-5 and elastin are shown in **Fig. 2**. Fibulin-5 was co-localized with elastic fibers. No apparent differences were observed between groups Y and O.

Messenger RNA expression analysis

The distribution of relative fibulin-5 expression is shown in **Fig. 3**. Median relative intensities of fibulin-5/GAPDH in groups Y, O, and C were 0.82 ± 0.43 , 0.90 ± 0.41 , and 0.76 ± 0.27 , respectively. Fibulin-5 mRNA expression was not significantly different between groups Y and O ($p = 0.97$).

Western blot analysis

The distribution of relative intensities of fibulin-5 is shown in **Fig. 4**. Median relative intensities in group Y, O, and C were 1.10 ± 0.44 , 1.64 ± 0.58 , and 2.05 ± 0.98 , re-

Fig. 3 Distribution of relative fibulin-5 expression. **Fig. 4** Distribution of relative intensities of fibulin-5.

spectively. The relative intensity in group Y was significantly lower than group O ($p = 0.006$).

Discussion

PSP typically occurs in tall and thin males between

the ages of 10 and 30 years and rarely occurs in persons over the age of 40 years.¹²⁾ Cigarette smoking increases the risk of PSP in men by as much as a factor of 20 in a dose-dependent manner.¹³⁾ Although patients with PSP do not have apparent lung disease, subpleural bullae are found in 48% to 100% of patients during video-assisted thoracoscopic surgery.⁵⁾ However, the mechanism of bulla formation remains unclear. It is reported that the development of blebs and bullae appear to be linked to a variety of factors, including distal airway inflammation,¹⁴⁾ hereditary predisposition,¹⁵⁾ ectomorphic physiognomy with more negative intrapleural pressures¹⁶⁾ and apical ischemia,¹⁷⁾ and abnormal connective tissue.^{18,19)} However, there are many patients with PSP without these risk factors. In these cases, the true cause of pneumothorax is still unknown.

The fibulins are a relatively recently recognized family of extracellular proteins that are widely expressed in the basement membranes of epithelia and blood vessels.⁷⁾ All fibulins have binding sites for other basement membrane proteins, such as fibrillin, fibronectin, proteoglycans, integrins, and tropoelastin. Fibulin-5 appears to be essential for the polymerization of elastin, because in mice and humans without functional copies of the fibulins-5 gene, tropoelastin is synthesized but is not assembled into its mature elastin fibrils.^{9,20,21)} Fibulin-5^{-/-} mice have loose skin hanging in folds, a short nose, and large cheeks. In addition to these changes, histological observation of the lung tissue revealed disruption of alveolar structure in adult fibulin-5^{-/-} lungs, which were most severe in the peripheral regions.^{9,20)}

We hypothesized that fibulin-5 reduction leads to fragility of lung tissue and development of blebs and bullae. First, we analyzed the clinical data to determine features of young patients with PSP. There were no apparent risk factors in patients with PSP except for smoking. All patients with pneumothorax who were older than 25 years were smokers. To determine these features and distinguish the influence of risk factors from age, patients were divided into 2 groups: younger than 25 years (group Y) and 25 years or older (group O). Our clinical data analysis showed that patients in group Y had a thin thorax compared with those in group O, although height and a/b ratio were not different between pneumothorax patients. These results suggested that young PSP patients have a long thorax rather than a thin thorax.

We then examined fibulin-5 protein levels and mRNA expression in pneumothorax patients and controls. We showed that fibulin-5 protein levels in group Y patients

were significantly lower than those in group O and C patients. On the other hand, fibulin-5 protein levels were not significantly different when they were compared between group O and C patients ($p = 0.14$). There was only 1 patient younger than 25 years in group C, and the protein level of fibulin-5 in this 14-years-old patient was high. These findings may indicate an association between fibulin-5 reduction and PSP in patients younger than 25 years. Since fibulin-5 is essential for elastogenesis, this indicates that the reduction of fibulin-5 protein results in a corresponding reduction in elastogenesis. However, mRNA expression of fibulin-5 was not significantly different among the groups. It is unclear why fibulin-5 protein was reduced in group Y without reduction of fibulin-5 mRNA expression.

Age-related macular degeneration is the most common cause of irreversible visual loss in the Western world.^{22,23)} Recently, missense mutations in fibulin-5 have been reported in association with age-related macular degeneration.²⁴⁾ Lotery et al. revealed that fibulin-5 secretion was significantly reduced for 4 age-related macular degeneration (p.G412E, p.G267S, p.I169 T, and p.Q124P) and 2 cutis laxa (p.S227P, p.C217R) mutations.²⁵⁾ They showed that some missense mutations lead to decreased fibulin-5 secretion with a possible corresponding reduction in elastinogenesis. To clarify the mechanism of reduction in fibulin-5 protein, we performed sequence analysis of fibulin-5. However, no sequence variations in fibulin-5 were found in pneumothorax patients.

In this study, there are some limitations with regard to samples. It was ethically difficult to obtain young healthy lung samples. Almost all patients who required surgery had pneumothorax or some congenital disorder and they were not suitable as controls. Therefore, we could obtain only 1 sample from a young healthy patient in this study.

In conclusion, we showed fibulin-5 reduction in patients with PSP younger than 25 years old. This is the first report of the association between PSP and fibulin-5. A long and thin thorax rather than a tall stature was also observed in young PSP patients. However, our results failed to clarify how fibulin-5 protein was reduced and the association between fibulin-5 reduction and chest deformity. Further investigation, e.g., investigation using animal models, will be required.

Acknowledgments

We are grateful to Dr. Yoshida, Dr. Nakai, Dr. Nakamura, Dr. Iwahashi, and Dr. Toguchi at Saiseikai

Wakayama Hospital for help with data collection. This study was also supported by clinical laboratory technicians at Saiseikai Wakayama Hospital, who helped with the accumulation of clinical samples.

Disclosure Statement

The authors report to *Annals of Thoracic and Cardiovascular Surgery* that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

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