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Differences in serum SP-D levels between German and Japanese subjects are associated with *SFTPD* gene polymorphisms

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Abstract

Background: Surfactant protein A (SP-A) and SP-D are clinically established in Japan as serum biomarkers for diagnosing interstitial lung diseases (ILDs). Serum SP-D levels are affected by genetic variants. We conducted the present study to examine whether serum SP-A and/or SP-D levels in healthy subjects (HS) and patients with ILDs differ between populations with different genetic backgrounds.

Methods: German subjects (n = 303; 138 patients with idiopathic interstitial pneumonias [IIPs] and 165 HS) and Japanese subjects (n = 369; 94 patients with IIPs and 275 HS) were enrolled. Serum SP-A and SP-D levels were measured using an enzyme-linked immunosorbent assay, and four single-nucleotide polymorphisms (SNPs) in the *SFTPD* gene were genotyped using genomic DNA extracted from blood samples.

Results: In both the German and Japanese cohorts, serum SP-A and SP-D levels were significantly higher in patients with IIPs than in HS. There were no significant differences in SP-A levels between the German and Japanese cohorts; however, we found that serum SP-D levels were significantly higher in the German cohort, both in patients with IIPs and in HS ($p < 0.001$ and $p = 0.005$, respectively). Furthermore, the genotype distributions of the four SNPs in the *SFTPD* gene (rs721917, rs1998374, rs2243639, and rs3088308) were significantly different between German and Japanese cohorts ($p < 0.001$, $p < 0.001$, $p = 0.022$, and $p < 0.001$, respectively), and univariate linear regression analyses revealed that the genotypes of rs721917, rs1998374, and rs2243639 significantly correlated with serum SP-D levels ($p < 0.001$, $p < 0.001$, and $p = 0.011$, respectively). Furthermore, multivariate analyses revealed that the genotypes of these three SNPs correlated independently with serum SP-D levels ($p < 0.001$, $p = 0.001$, and $p = 0.038$, respectively), whereas ethnicity did not significantly correlate with serum SP-D levels.

Conclusions: In patients with IIPs and HS, serum SP-D, but not SP-A, levels were significantly higher in the German than in the Japanese cohort, in part, because of the different frequencies of *SFTPD* gene polymorphisms.

Keywords: Biological marker, Idiopathic interstitial pneumonia, Single nucleotide polymorphism, Surfactant protein-A (SP-A), Surfactant protein-D (SP-D)

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Background

Idiopathic interstitial pneumonias (IIPs) are a group of diffuse parenchymal lung diseases characterized by interstitial involvement resulting from various patterns of inflammation and fibrosis of unknown cause. The prevalence of IIPs has been generally reported as 5–20 per 100,000 persons [1-4], with a recommendation that the diagnosis of IIPs be made according to clinical history, physical findings, chest radiographs, and/or lung function tests [5,6]. However, some patients may not complain of symptoms or present with abnormal chest radiographs and/or lung function tests, even though they already suffer from IIPs. Therefore, the availability of diagnostic tools that can discriminate patients with IIPs from healthy subjects (HS) at an early stage will be undoubtedly useful in clinical practice. In this regard, serum biomarkers draw particular interest because they are easy to obtain from patients.

Pulmonary surfactant protein A (SP-A) and SP-D are water-soluble proteins derived mainly from type II pneumocytes and belong to the collectin subgroup of the C-type lectin superfamily [7]. Because one of the key histological feature of the lung affected with interstitial lung diseases (ILDs) involves injury and/or regeneration of Type II pneumocytes [8], soluble proteins derived from Type II pneumocytes, such as SP-A, SP-D, and Krebs von den Lungen 6 (KL-6), have been studied as potential biomarkers for ILDs [9-15]. These biomarkers can be useful for early detection of ILDs, predicting disease outcome, and monitoring the clinical course [16-18]. On the basis of these findings, serum SP-A, SP-D, and KL-6 have been clinically approved by Japan's Health Insurance Program as diagnostic markers for ILDs in 1999, and more than 2,000,000 samples of these biomarkers are now examined yearly in Japan. However, in most countries, assays for these biomarkers are limited to research and are currently unavailable for clinical practice.

We recently conducted an international study to measure the serum levels of KL-6 and analyze the rs4072037 genotypes of Mucin 1 (*MUC1*) in German and Japanese cohorts that included patients with ILDs and healthy subjects. We demonstrated that the cutoff value of KL-6 that discriminated patients with ILDs from HS was significantly higher in the German than in the Japanese cohort because of differences in the distribution of rs4072037 genotypes between them [19]. The correlations between rs4072037 genotypes and serum KL-6 levels have also been demonstrated in a Dutch cohort [20]. Moreover, serum SP-D levels were found to be correlated with genetic polymorphisms of surfactant protein D (*SFTPD*) [21-23]. According to the International HapMap project [24], the genotype distributions of some single-nucleotide polymorphisms (SNPs) in the *SFTPD* gene differ according to ethnicities. We hypothesized, therefore, that differences exist in serum SP-D and/or SP-A levels between different ethnic populations. To test this hypothesis, we first measured serum SP-A and SP-D levels and compared them between German and Japanese cohorts that included patients with IIPs and healthy subjects. Next, we evaluated the correlations between serum SP-D levels and *SFTPD* gene polymorphisms in the German and Japanese cohorts.

Methods

Study subjects

Between February 2007 and December 2011, 138 consecutive German-Caucasian patients with IIPs at Ruhrlandklinik, University Hospital Essen (Essen, Germany) and 94 Japanese patients with IIPs at Hiroshima University Hospital (Hiroshima, Japan) were enrolled in the present study. 165 German-Caucasian and 275 Japanese HS were recruited from the subjects who visited these hospitals to undergo health checkup. Diagnoses of IIPs were made according to the criteria of the American Thoracic Society (ATS)/European Respiratory Society (ERS) published in 2002 [5]. We excluded patients with ILDs of known cause, including drugs, collagen vascular diseases, and hypersensitivity pneumonia. Each HS underwent pulmonary function tests and chest X-ray studies, and we excluded those with apparent lung disease such as ILDs or chronic obstructive pulmonary disease (COPD). The Ethics Committees of Ruhrlandklinik (IRB 06-3170) and Hiroshima University Hospital (IRB 326) approved this study, which was conducted in accordance with the ethical standards established by the Helsinki Declaration of 1975. All patients and healthy volunteers provided written informed consent to participate in the study and permission to use their samples.

Lung function values

Physiologic assessment included measurements of thoracic gas volume, total lung capacity, forced vital capacity (FVC), forced expiratory volume in one second (FEV1), and single-breath diffusing capacity of the lung for carbon monoxide (DL_{CO}) as previously described [25-28]. The protocol for lung function measurements conformed to ATS recommendations [29].

Measurement of serum SP-A and SP-D levels

Serum samples were obtained from 234 patients with IIPs and 440 HS and stored at -80°C. Serum SP-A and SP-D levels were measured using a sandwich-type enzyme-linked immunosorbent assay (ELISA) by using commercially available ELISA kits (SP-A test Kokusai-F kit, Sysmex Corporation Kobe, Japan; SP-D kit YAMASA EIA II, Yamasa Corporation Choshi, Japan) as previously described [28,30].

DNA preparation and SNP genotyping

We extracted DNA from peripheral whole venous blood samples by using the phenol-chloroform extraction and ethanol precipitation methods as previously described [31]. SNPs only in the *SFTPD* gene were investigated because difference in serum levels between German and Japanese was just observed for SP-D but not for SP-A. We analyzed HapMap genotyping data [24] by using chi-square tests and picked up 18 SNPs in the *SFTPD* gene that showed significantly different genotype distributions between CEU (Utah residents with ancestry from northern and western Europe) and JPT (Japanese in Tokyo, Japan). From these 18 SNPs, we selected four SNPs (rs721917, rs1998374, rs2243639, and rs3088308; Figure 1) that have been reported to be associated with serum SP-D levels [21-23] and/or TaqMan SNP Genotyping Assays are available for. SNP genotyping was carried out by using TaqMan SNP Genotyping Assay C 1362980-10, C 12124514-10, C 26726205-10, and C 26726209-10 (Life Technologies Corp. Carlsbad, California, USA) and the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies Corp. Carlsbad, California, USA).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Data for individual variables for 2 groups were analyzed using the Mann–Whitney *U*-test or the chi-square test. The significance levels for multiple pairwise comparisons were set according to Bonferroni's correction. Receiver operating characteristic (ROC) curves were generated to assess the diagnostic abilities of SP-A and SP-D. Linear regression analyses were conducted to study the correlations between serum SP-D levels and each of the four SNPs. If the correlation was statistically significant, multivariate regression analysis was performed to determine whether the SNPs in the *SFTD* gene independently affect serum SP-D levels even when adjusted by the covariates as follows: age, ethnicity, and case-control status. All statistical analyses were performed using SPSS for Windows, version 18.0 (SPSS Inc. Chicago, USA).

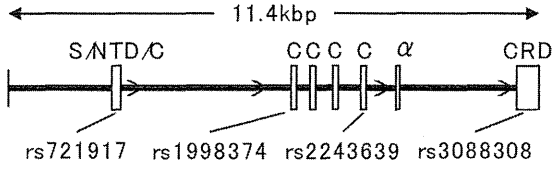

Figure 1 Functional features of the SFTPD gene. The thick horizontal bar represents the intronic region, and the white boxes represent exons. S, signal peptide; NTD, N-terminal domain; C, collagen domain; α, αhelical neck region; CRD, carbohydrate recognition domain.

Results

Serum SP-D but not SP-A levels were significantly higher in the German cohort

The clinical characteristics of 232 patients with IIPs (138 German and 94 Japanese) and 440 HS (165 German and 275 Japanese) are shown in Table 1. Serum SP-A levels in German HS, German patients with IIPs, Japanese HS, and Japanese patients with IIPs were 29.7 ± 1.1 ng/ml, 79.4 ± 3.3 ng/ml, 29.4 ± 0.9 ng/ml, and 83.0 ± 6.3 ng/ml, respectively (Figure 2A). Serum SP-D levels in German HS, German patients with IIPs, Japanese HS, and Japanese patients with IIPs were 59.8 ± 2.6 ng/ml, 373.3 ± 20.3 ng/ml, 39.9 ± 1.6 ng/ml, and 323.9 ± 29.8 ng/ml, respectively (Figure 2B). In both the German and the Japanese cohort, the serum SP-A and SP-D levels in patients with IIPs were significantly higher than those in HS. Further, serum SP-D levels in German HS were significantly higher than those in Japanese HS ($p < 0.001$; Figure 2B), and serum SP-D levels in German patients with IIPs were significantly higher than those in Japanese patients with IIPs ($p = 0.005$; Figure 2B). In contrast, there was no significant difference in serum SP-A levels between the cohorts, within the HS and patients with IIPs ($p = 0.677$ for HS and $p = 0.326$ for patients with IIPs; Figure 2A).

To evaluate the ability of the serum levels of SP-A and SP-D to discriminate patients with IIPs from HS, ROC curves were drawn for each cohort. The areas under the curves (AUCs) for serum SP-A in the German (AUC = 0.940, 95% confidence interval [CI] = 0.915–0.966; $p < 0.001$) and Japanese cohorts (AUC = 0.902, 95% CI = 0.868–0.936; $p < 0.001$) were sufficiently high to distinguish patients with IIPs from HS (Figure 2C). This was true for the AUCs for serum SP-D levels in the German (AUC = 0.977, 95% CI = 0.957–0.996; $p < 0.001$) and Japanese cohorts as well (AUC = 0.973, 95% CI = 0.952–0.994; $p < 0.001$; Figure 2D).

Distributions of SFTPD gene polymorphisms are not different between patients with IIPs and healthy subjects

DNA was extracted from blood samples from 139 of 303 German (102 patients with IIPs and 37 HS) and 338 of 369 Japanese subjects (63 patients with IIPs and 275 HS). Because collection of whole blood for DNA extraction was missed or refused, we could not obtain the genotype data in a part of German patients with IIPs, German HS, and Japanese patients with IIPs. We compared subject characteristics including serum SP-A and SP-D levels based on the availability of genotype data and found that there was no significant difference in serum SP-A or SP-D levels between the subjects with and without genotype data in German patients with IIPs, German HS, or Japanese patients with IIPs (Additional file 1: Tables S1A and S1B). The genotype distributions

Table 1 Characteristics of study subjects

	German	Japanese	p value
Patients with IIPs			
Number of the subjects	138	94	
Age (years)	67.4 ± 0.8	68.0 ± 1.0	0.982
Gender (male/female)	88 (63.8%)/50 (36.2%)	64 (68.1%)/30 (31.9%)	0.497
Smoking (Cu/Ex/Non/ND)	14 (10.1%)/54 (39.1%)/ 64 (46.4%)/6 (4.4%)	11 (11.7%)/46 (49.0%)/ 35 (37.2%)/2 (2.1%)	0.297
VC (percent predicted)	66.1 ± 1.6	70.3 ± 2.2	0.163
DL_{CO} (percent predicted)	47.7 ± 1.6	44.8 ± 1.8	0.255
Diagnostic category (IPF/NSIP)	94 (68.1%)/44 (31.9%)	61 (64.9%)/33 (35.1%)	0.609
Healthy subjects			
Number of the subjects	165	275	
Age (years)	36.5 ± 0.9	49.8 ± 0.4	< 0.001
Gender (male/female)	60 (36.4%)/105 (63.6%)	227 (82.5%)/48 (17.5%)	< 0.001
Smoking (Cu/Ex/Non/ND)	41 (24.8%)/20 (12.1%)/ 90 (54.6%)/14 (8.5%)	82 (29.8%)/62 (22.6%)/ 131 (47.6%)/0 (0.0%)	0.026

Data are shown as mean ± SEM.
Statistical significance was tested by Mann-Whitney *U*-test or Chi-square test.
Cu, Current smoker; *Ex*, Ex-smoker; *Non*, Non-smoker; *ND*, No data; *IIPs*, Idiopathic interstitial pneumonias; *VC*, Vital capacity; DL_{CO}, Diffusing capacity of the lung for carbon monoxide; *IPF*, Idiopathic pulmonary fibrosis; *NSIP*, Nonspecific interstitial pneumonia.

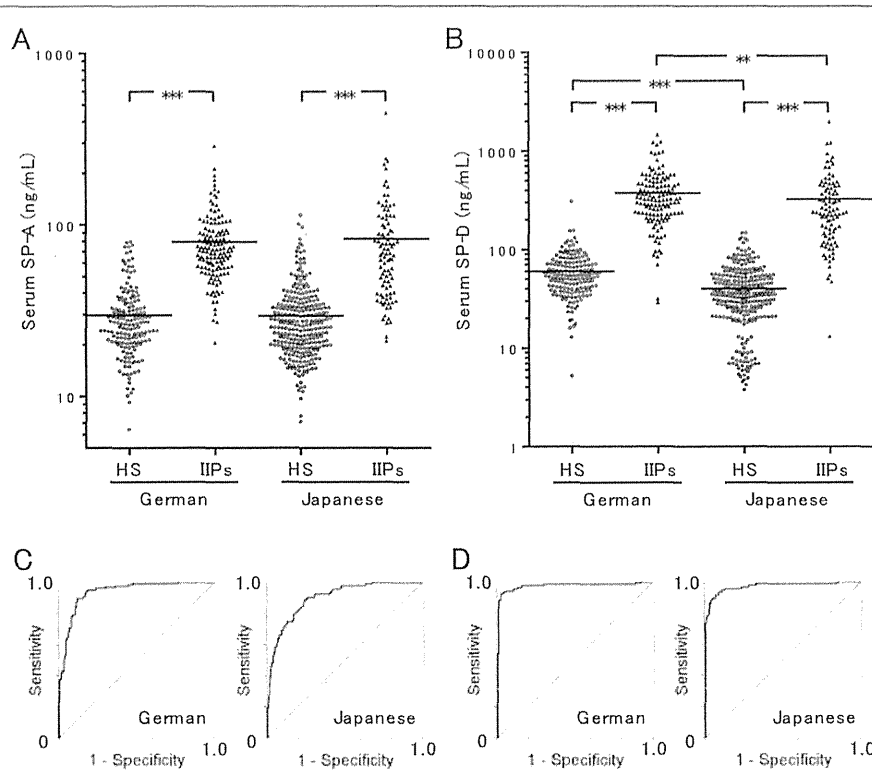

Figure 2 Comparison of serum SP-A and SP-D levels between the cohorts. (A) Serum SP-A and **(B)** SP-D levels in German and Japanese cohorts. Receiver operating characteristic (ROC) curves of **(C)** serum SP-A and **(D)** SP-D in German (left panel) and Japanese (right panel) cohorts. The horizontal bars represent the mean values. The significance level was defined $\alpha = 0.013$ (four comparisons in four groups). ***$p < 0.001$, **$p < 0.013$ (Mann-Whitney *U*-test).

of the four SNPs were in Hardy–Weinberg equilibrium in German patients with IIPs, German HS, Japanese patients with IIPs, and Japanese HS (Table 2). As shown in Table 2, the genotype distributions of the four SNPs did not differ between patients with IIPs and HS in both the German and Japanese cohorts. Further, the four SNPs (rs721917, rs1998374, rs2243639, and rs3088308) showed significantly different genotype distributions between the German and Japanese cohorts ($p < 0.001$, $p < 0.001$, $p = 0.022$, and $p < 0.001$, respectively; Additional file 2: Table S2).

Serum SP-D levels differ according to SFTPD gene polymorphisms

Within each German and Japanese cohort, serum SP-D levels were compared based on the genotypes of each SNP in the *SFTPD* gene. Within all genotypes of the four SNPs, the serum SP-D levels of patients with IIPs were significantly higher than those of HS in both German and Japanese cohorts (Figure 3). Furthermore, serum SP-D levels of Japanese HS were significantly higher in the rs721917 T/T than in the T/C cohort ($p < 0.001$) and were also significantly higher in the T/C than in the C/C cohort ($p < 0.001$; Figure 3A). Moreover, serum SP-D levels of patients with IIPs tended to be higher in the rs721917 T/T and be lower in the C/C cohort in both German and Japanese populations (Figure 3A). Similarly, serum SP-D levels also showed a trend to be different between the genotypes of rs1998374 (Figure 3B) and rs2243639 (Figure 3C). In contrast, serum SP-D levels did not differ according to the rs3088308 genotypes (Figure 3D).

Correlation between serum SP-D levels and SFTPD gene polymorphisms is statistically independent

Linear regression analyses were performed to further examine whether the *SFTD* gene polymorphisms affect serum SP-D levels independently from other covariates. Univariate analyses revealed that the rs721917, rs1998374, and rs2243639 genotypes correlated significantly with serum SP-D levels ($p < 0.001$, $p < 0.001$, and $p = 0.011$, respectively), but not that of rs3088308 (Table 3). We next performed multivariate analysis, including age, ethnicity, and case-control status (patients with IIPs vs HS) as covariates, because these covariates showed significant

Table 2 Genotype distributions of single nucleotide polymorphisms in SFTPD gene - patients with IIPs vs HS -

	German					Japanese				
rs721917	Total	C/C	T/C	T/T	HWE	Total	C/C	T/C	T/T	HWE
Patients with IIPs	102	25	47	30	0.862	63	19	30	14	0.973
		(24.5%)	(46.1%)	(29.4%)			(30.2%)	(47.6%)	(22.2%)	
Healthy subjects	37	9	11	17	0.266	275	95	136	44	0.960
		(24.3%)	(29.7%)	(46.0%)			(34.5%)	(49.5%)	(16.0%)	
Chi-square test				$p = 0.140$					$p = 0.476$	
rs1998374	Total	C/C	T/C	T/T	HWE	Total	C/C	T/C	T/T	HWE
Patients with IIPs	102	3	18	81	0.655	63	6	35	22	0.561
		(2.9%)	(17.7%)	(79.4%)			(9.5%)	(55.6%)	(34.9%)	
Healthy subjects	37	0	5	32	0.913	275	57	125	93	0.679
		(0.0%)	(13.5%)	(86.5%)			(20.7%)	(45.5%)	(33.8%)	
Chi-square test				$p = 0.464$					$p = 0.102$	
rs2243639	Total	C/C	T/C	T/T	HWE	Total	C/C	T/C	T/T	HWE
Patients with IIPs	102	43	45	14	0.960	63	28	28	7	1.000
		(42.2%)	(44.1%)	(13.7%)			(44.4%)	(44.4%)	(11.1%)	
Healthy subjects	37	13	15	9	0.741	275	140	113	22	0.996
		(35.1%)	(40.6%)	(24.3%)			(50.9%)	(41.1%)	(8.0%)	
Chi-square test				$p = 0.324$					$p = 0.563$	
rs3088308	Total	A/A	A/T	T/T	HWE	Total	A/A	A/T	T/T	HWE
Patients with IIPs	102	86	16	0	0.711	63	59	4	0	0.968
		(84.3%)	(15.7%)	(0.0%)			(93.7%)	(6.3%)	(0.0%)	
Healthy subjects	37	32	5	0	0.913	275	265	10	0	0.955
		(86.5%)	(13.5%)	(0.0%)			(96.4%)	(3.6%)	(0.0%)	
Chi-square test				$p = 0.752$					$p = 0.330$	

IIPs, Idiopathic interstitial pneumonias; *HWE*, Hardy-Weinberg equilibrium.

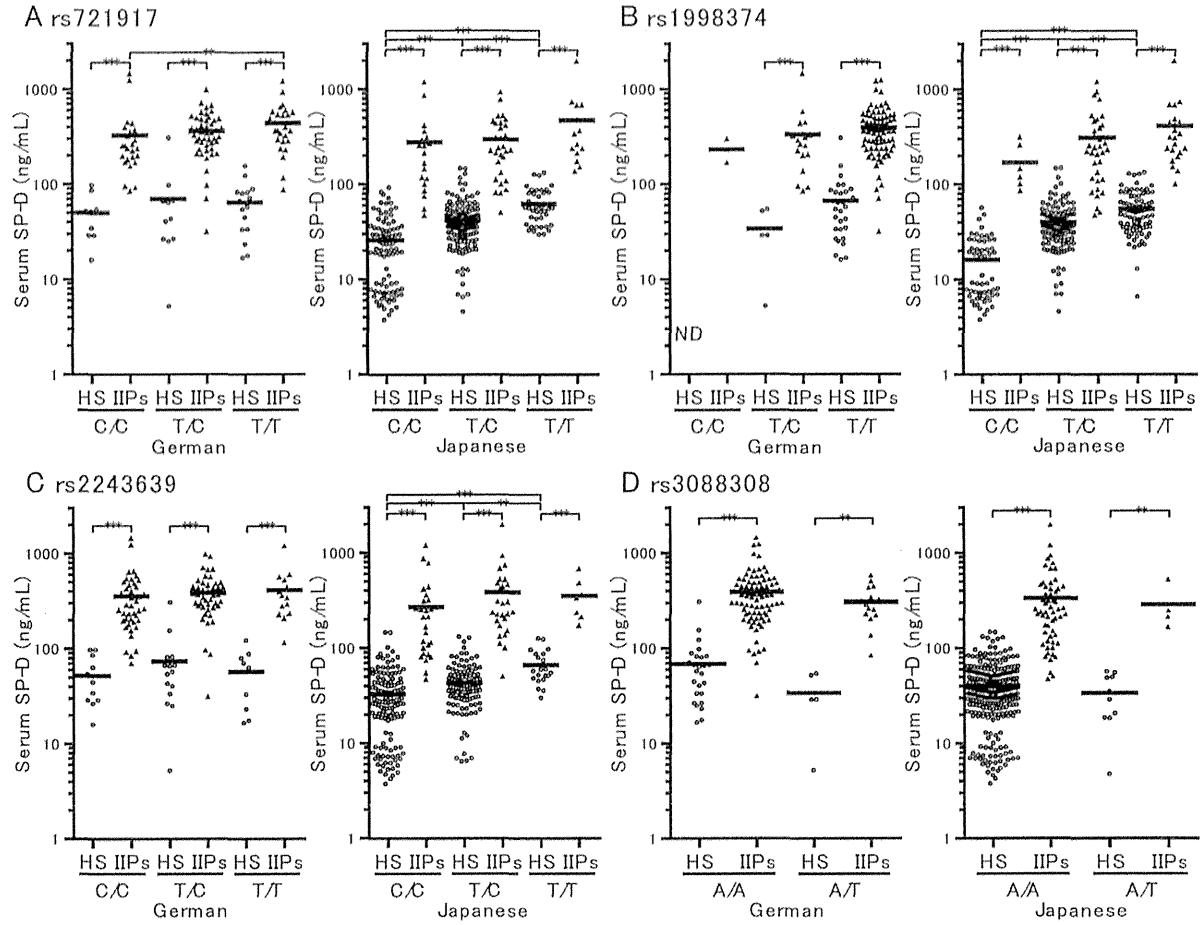

Figure 3 Relationship between genotype and serum SP-D levels. Serum SP-D levels were compared between each genotype: **(A)** rs721917, **(B)** rs1998374, **(C)** rs2243639, and **(D)** rs3088308. For each genotype, data of the German cohort are shown in the left and those of the Japanese cohort are shown in the right. The horizontal bars represent the mean values. **(A)**, **(B)**, **(C)** The significance level was defined as $\alpha = 0.006$ (nine comparisons in six groups). ***$p < 0.001$, **$p < 0.006$ (Mann-Whitney *U*-test). **(D)** The significance level was defined as $\alpha = 0.0125$ (four comparisons in four groups). ***$p < 0.001$, **$p < 0.013$ (Mann-Whitney *U*-test).

correlations with serum SP-D levels in univariate analyses ($p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). The rs721917, rs1998374, and rs2243639 genotypes significantly correlated with serum SP-D levels in multivariate analyses ($p < 0.001$, $p = 0.001$, and $p = 0.038$, respectively) as well. Further, among the three covariates, only the case-control status revealed significant correlations with serum SP-D levels in the three multivariate models ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively; Table 3), although age and ethnicity did not significantly correlate with serum SP-D levels.

Discussion

In the present study, we demonstrated that serum SP-A and SP-D levels were significantly higher in patients with IIPs than in HS in both German and Japanese subjects. Moreover, we found that in patients with IIPs and HS, serum SP-D levels were significantly higher in the German than in the Japanese cohort, whereas there were no significant differences in serum SP-A levels between the two cohorts. The genotype distributions of SNPs in the *SFTPD* gene, which affect the serum SP-D levels, differed between the cohorts. Furthermore, multivariate analyses demonstrated that there were statistically independent correlations between serum SP-D levels and the rs721917, rs1998374, and rs2243639 genotypes of the *SFTPD* gene, regardless of ethnicity and presence of IIPs.

The significantly higher serum SP-A and SP-D levels in patients with IIPs compared with those of the German HS suggest their utility as diagnostic biomarkers for IIPs, even in the German population. Serum SP-A levels in the German patients with IIPs or HS were comparable to those in their Japanese counterparts (Figure 2A); however,

Table 3 Correlations between serum SP-D levels and SFTPD gene polymorphisms

	Variable	Regression coefficient (95% CI.)	p value	VIF
rs721917	**Univariate model**			
	C/C vs T/C vs T/T	55.276 (28.473 - 82.079)	< 0.001	
	Multivariate model			
	C/C vs T/C vs T/T	35.149 (15.341 - 54.957)	< 0.001	1.031
	Age	−0.444 (−2.009 - 1.121)	0.578	1.978
	Japanese vs German	24.061 (−13.125 - 61.246)	0.204	1.437
	HS vs patients with IIPs	307.740 (260.743 - 354.736)	< 0.001	2.515
rs1998374	**Univariate model**			
	C/C vs T/C vs T/T	86.550 (59.832 - 113.269)	< 0.001	
	Multivariate model			
	C/C vs T/C vs T/T	36.172 (14.289 - 58.056)	0.001	1.203
	Age	−0.353 (−1.918 - 1.213)	0.658	1.972
	Japanese vs German	11.114 (−28.165 - 50.393)	0.578	1.598
	HS vs patients with IIPs	304.645 (257.602 - 351.689)	< 0.001	2.511
rs2243639	**Univariate model**			
	C/C vs T/C vs T/T	38.179 (8.965 - 67.393)	0.011	
	Multivariate model			
	C/C vs T/C vs T/T	22.707 (1.310 - 44.104)	0.038	1.017
	Age	−0.360 (−0.449 - 0.654)	0.654	1.976
	Japanese vs German	29.314 (−8.000 - 66.627)	0.123	1.423
	HS vs patients with IIPs	306.421 (259.045 - 353.798)	< 0.001	2.514
rs3088308	**Univariate model**			
	A/A vs A/T	37.750 (−37.732 - 113.231)	0.326	

The genotypes of each single nucleotide polymorphism are arranged in the order of lowest to highest serum SP-D levels.
CI, Confidence interval; *HS*, Healthy subjects; *IIPs*, Idiopathic interstitial pneumonias; *VIF*, Variance inflation factor.

the serum SP-D levels in German patients with IIPs or HS were significantly higher than those in their Japanese counterparts (Figure 2B). These results imply that serum SP-D levels are affected by the different ethnicity, whereas serum SP-A levels are not. In agreement with our present study results, serum SP-D levels in Caucasian HS were reported to be higher than those in Asian HS [32].

To explain the differences in serum SP-D levels between the German and Japanese cohorts, we determined the relationship between serum SP-D levels and genotypic differences in the *SFTPD* gene. We found that among the SNPs in *SFTPD* gene, rs721917, rs1998374, and rs2243639, but not rs3088308, affected the serum SP-D levels (Figure 3), and the distributions of these polymorphisms were different between the German and Japanese cohorts. SP-A and SP-D molecules comprise an N-terminal domain (NTD), a collagen domain, an α-helical neck region, and a carbohydrate recognition domain (CRD; Figure 1) [33]. SP-D polypeptide chains bind together through interpolypeptide disulfide bonds in the NTD to form oligomers, and the degree of oligomerization is affected by the genotypes of coding SNP rs721917 in the NTD. Thus, the T/T and C/C genotypes correlate with higher and lower-order oligomers, respectively [33-36]. These structural variations of SP-Ds might affect their serum levels, and our results demonstrate that serum SP-D levels differed according to the rs721917 genotype (Figure 3A). Furthermore, we also found that serum SP-D levels were affected by the genotypes of rs1998374 and rs2243639, both of which are located in the collagen domain (Figures 1, 3B and C), but they were not affected by the genotypes of rs3088308, the coding SNP in the CRD (Figures 1 and 3D). These findings suggest the possibility that the collagen domain is also associated with the degree of oligomerization of the SP-D molecule.

To determine whether serum SP-D levels were independently correlated with *SFTPD* gene polymorphisms and/or ethnicity, we performed multivariate regression analyses and found that the correlations between the genotypes of three SNPs in the *SFTPD* gene and serum SP-D levels remained statistically significant in the multivariate models (Table 3). In contrast, the correlations between ethnicity and serum SP-D levels were insignificant (Table 3). These findings suggest that serum SP-D levels are more strongly affected by *SFTPD* gene

polymorphisms than by ethnicity. Therefore, the difference in serum SP-D levels that were observed between German and Japanese cohorts might be partially explained by the differences in the frequencies of *SFTPD* gene polymorphisms between the cohorts.

In contrast, we found that the genotype distributions of four SNPs in the *SFTPD* gene did not differ between patients with IIPs and HS in both cohorts (Table 2). SP-A and SP-D play important roles in surfactant-related functions and in host defense against inhaled pathogens [37]. Moreover, *SFTPD* gene polymorphisms, rs721917 in particular, have been reported to correlate with susceptibility to COPD, community-acquired pneumonia, ILDs, and lung cancer [23,38-41]. As discussed above, rs721917 is known to be associated with the degree of oligomerization of the SP-D molecules [33-36]. This difference in oligomerization might affect the surfactant and/or host defense functions of SP-D and thus correlate with susceptibility to various respiratory diseases. In the present study, however, no significant correlation between *SFTPD* gene polymorphisms and susceptibility to IIPs was demonstrated. We believe that a larger sample size of study is needed to determine the correlations between *SFTPD* gene polymorphisms and susceptibility to IIPs.

We are aware that there are some limitations in this study. First, age, gender, and smoking status were significantly different between German HS and Japanese HS because the populations who undergo health checkup were different between Germany and Japan. We performed linear regression analysis to assess the interference between these factors and serum SP-D levels, and we found a significant correlation between age and serum SP-D levels. Thus, we included age into the multivariate analyses and confirmed that our results were significant and independent of age differences. Second, the number of subjects available for genomic analyses was relatively small. Third, only German and Japanese populations were studied. It remains unclear whether the findings of the present study can be applied to other ethnic groups such as African Americans.

Conclusions

In conclusion, we demonstrated that the serum levels of SP-A and SP-D were significantly elevated in patients with IIPs in the German and Japanese cohorts, whereas serum SP-D but not SP-A levels were significantly higher in the German cohort. We have explained the differences in serum SP-D levels between these cohorts, at least in part, by the different frequencies of *SFTPD* gene polymorphisms. Although we believe that these data are compelling, further investigations with larger number of subjects are required to assess the utility of serum SP-A and SP-D in non-Japanese cohorts.

Additional files

Additional file 1: Table S1. Comparisons of the baseline characteristics including serum SP-A and SP-D levels between the subjects with and without genotype data in the German (Table S1A) and Japanese (Table S1B) cohorts.

Additional file 2: Table S2. Comparisons of the genotype distributions of single nucleotide polymorphisms in *SFTPD* gene between the German and Japanese cohorts.

Abbreviations

IIP: Idiopathic interstitial pneumonia; SP-A: Surfactant protein A; SP-D: Surfactant protein D; ILD: Interstitial lung disease; KL-6: Krebs von den Lungen 6; MUC1: Mucin 1; SFTPD: Surfactant protein D; SNP: Single nucleotide polymorphism; HS: Healthy subjects; ATS: American Thoracic Society; ERS: European Respiratory Society; COPD: Chronic obstructive pulmonary disease; FVC: Forced vital capacity; FEV1: Forced expiratory volume in one second; DL_{CO}: Single-breath diffusing capacity of the lung for carbon monoxide; ELISA: Enzyme-linked immunosorbent assay; ROC: Receiver operating characteristic; AUC: Area under the curve; CI: Confidence interval; NTD: N-terminal domain; CRD: Carbohydrate recognition domain.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YH drafted and finalized the manuscript, performed part of the serum measurement, extraction of DNA, genotyping, and statistical analyses. NH, NI, NK and UC conceived the study, and participated in its design and coordination and helped to draft and finalize the manuscript. ST performed part of the extraction of DNA and genotyping. FB, JG and SO recruited the study subjects, ascertained diagnosis, and helped to draft and finalize the manuscript. All authors read and approved the final manuscript.

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Case report

Two patients with new granulomatous lung lesions during treatment of Crohn's disease

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ABSTRACT

Two patients with granulomatous lung lesions thought to be related to Crohn's disease (CD) are reported. Patient 1 was a 43-year-old man who was diagnosed with CD at age 11 years. He developed a fever in the 38 °C, and a chest X-ray and CT scan showed infiltrates with air bronchograms in the right upper lobe and left lingular segment. Transbronchial lung biopsy (TBLB) revealed granulomatous lesions. Patient 2 was a 76-year-old woman who was diagnosed with CD at age 44 years. Chest CT showed infiltrates and nodular shadows in both lung fields. Video-assisted thoracoscopic surgery (VATS) in June 2012 revealed granulomatous lesions. Tuberculosis, fungal infections, drug-induced lung disorder, and sarcoidosis were ruled out as a cause of the granulomatous lesions in both patients. The aetiology was thought to be CD.

1. Introduction

Crohn's disease (CD) is a granulomatous inflammatory disease of unknown aetiology, but thought to involve abnormal immune function, with a predilection to develop in the small and large intestines [1,2]. Granulomatous lung lesions in CD have not previously been reported in Japan, and only 3 cases have been reported overseas [3]. Two rare cases of CD-related granulomatous lung lesions are reported, and the relevant literature is discussed.

2. Case reports

2.1. Patient 1

Patient 1 was a 43-year-old man who was diagnosed with CD in 1979 (age 11 years). He had been followed as an outpatient by the Department of Gastroenterology at our hospital. He underwent an ileocoecal resection in 1981 (age 13 years), partial small bowel resection in 1987 (age 19 years), and transverse colon stricturplasty in 1999 (age 31 years). Parenteral nutrition (elemental diet: Elental, Racol) was then started. Mesalazine (5-ASA) was started in 2006, and infliximab (an anti-TNF-α antibody drug) was started in 2007 (age 39 years). However, he continued to have severe active CD, and adalimumab (another anti-TNF-α antibody drug) was started in March 2011 (age 42 years). The patient's gastrointestinal symptoms were controlled, but he developed a dry cough in mid-November 2011 (age 43 years), followed by a fever (38 °C) in early December 2011, and he was evaluated by our department.

A chest X-ray and CT showed bilateral infiltrates with air bronchograms (Fig. 1). The patient was diagnosed with community-acquired pneumonia (WBC 4300/μL, CRP 0.07 mg/dL), and antibiotic therapy with ceftriaxone (CTRX) was prescribed. However, since the dry cough did not improve, the patient was admitted to our department in mid-December 2011 for further evaluation and treatment. Bronchoscopy was performed, and transbronchial lung biopsy (TBLB) of the right upper lobe revealed a non-caseating granuloma with multi-nucleated giant cells (Fig. 2).

As testing for the aetiology of the granulomatous lesions, a QuantiFERON-TB test was negative, a tuberculin reaction was negative, and acid-fast staining of the bronchoscopy specimens (bronchial lavage fluid, TBLB) was negative. Thus, tuberculosis was unlikely. Grocott staining, β-D glucan, and cryptococcal antigen

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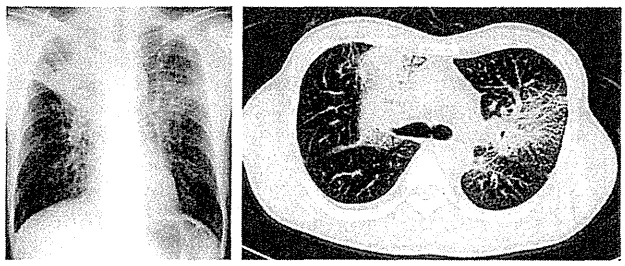

Fig. 1. Chest X-ray (left) and CT (right) taken in December 2011 show infiltrative shadows in the bilateral upper lobes.

testing of the TBLB specimens were negative, so a fungal infection was also unlikely. Aggressive therapy was not considered necessary, so the patient was followed as an outpatient. On a chest X-ray taken in July 2012, and on chest CT in August 2012, the infiltrate mainly in the right upper lobe had spontaneously disappeared (Fig. 3).

2.2. Patient 2

Patient 2 was a 76-year-old woman who was diagnosed with small intestinal CD in 1980 (age 44 years). She has been followed as an outpatient by the Department of Gastroenterology at our hospital. Salazosulfapyridine (5-ASA) was started in 1985 (age 49 years). She underwent a small bowel strictureplasty in 1992 (age 56 years), ileocoecal resection in 2003 (age 67 years), and then parenteral nutrition (Elental) was started.

The patient's gastrointestinal symptoms were well controlled, but she began to lose weight in September 2011 (10 kg weight loss/6 months). A CT scan in December 2011 showed thickened bronchiolar walls with multiple nodular shadows in peripheral bronchi of both lung fields, and bronchoscopy was performed in February 2012. The bronchoalveolar lavage (BAL) fluid showed predominant lymphocytosis (57%) and a CD4/CD8 ratio of 0.94. TBLB revealed no significant findings.

Chest X-ray and CT in May 2012 showed new infiltrates in the right lower and left upper lobes (Fig. 4). In June 2012, Video-assisted thoracoscopic surgery (VATS) of the lingula was performed, and histopathology showed an epithelioid cell granuloma with giant cells (Fig. 5). Since acid-fast cultures of the bronchial

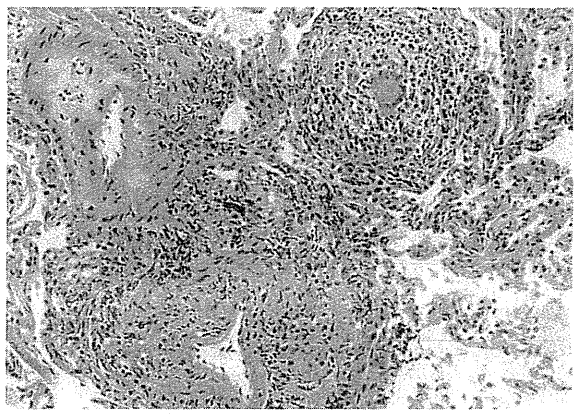

Fig. 2. Photomicrogram of transbronchial lung biopsy reveals non-caseating granuloma with multi-nucleated giant cells and lymphocytic infiltrate (haematoxylin-eosin, × 140).

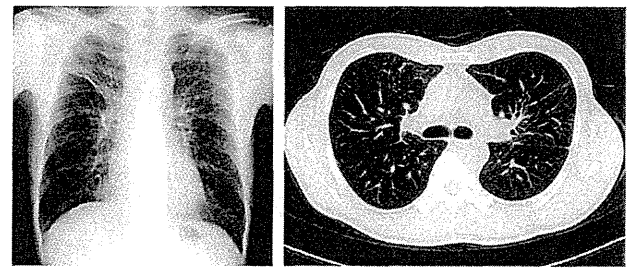

Fig. 3. Chest X-ray (left) and CT (right) taken in August 2012. The infiltrative shadow noted in Fig. 1 has disappeared spontaneously.

lavage fluid and lung biopsy tissue were negative, a mycobacterial infection was unlikely. Grocott staining, β-D glucan, and cryptococcal antigen testing were negative, so a fungal infection was also unlikely. Serum ACE was not elevated, the tuberculin reaction was negative, and the pulmonary hilar lymph nodes were not enlarged; thus, sarcoidosis was also ruled out. Drug treatment had not been switched during outpatient follow-up, so drug-induced pneumonia was also unlikely.

These findings were consistent with CD-related pulmonary lesions based on a diagnosis by exclusion and the histopathology. The patient developed dyspnoea on exertion, and treatment with tapering doses of prednisolone (PSL) starting at a dose of 40 mg/day was begun in July 2012. The exertional dyspnoea and imaging findings improved rapidly (Fig. 6).

3. Discussion

Crohn's disease (CD) was originally called regional ileitis and was first reported by Burrill B. Crohn MD at Mount Sinai Hospital (United States) in 1932 [4]. CD is characterized by chronic inflammatory granulomatous lesions of unknown aetiology that are associated with ulcerations and fibrosis. CD usually presents in younger patients with oedema and ulcerations in the small and large intestines, and intestinal strictures and fistulas often develop. CD most commonly affects the terminal ileum, but any site in the gastrointestinal tract may be involved [5]. Extra-intestinal complications of CD include joint complications (ankylosing spondylitis, sacroiliitis, peripheral arthritis), skin complications (erythema nodosum, pyoderma gangrenosum), ocular complications (episcleritis, scleritis, uveitis), hepatobiliary complications (primary sclerosing cholangitis), and pulmonary complications (organizing pneumonia) [6].

When granulomatous lesions develop in CD patients, granulomatous infections such as mycobacterial or fungal infections, drug-induced pneumonia, and sarcoidosis must be included in the differential diagnosis. Granulomas in CD are sarcoid-like granulomas,

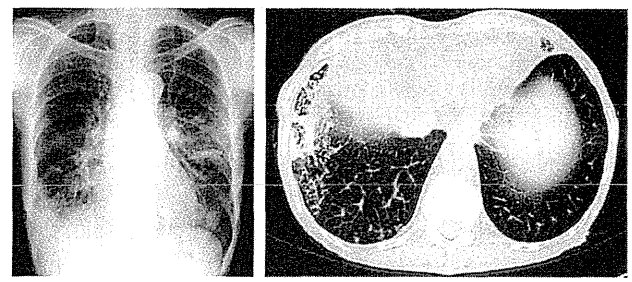

Fig. 4. Chest X-ray (left) and CT (right) taken on admission May 2012 reveals infiltrative and small nodular shadow in the right lower and left upper lobes.

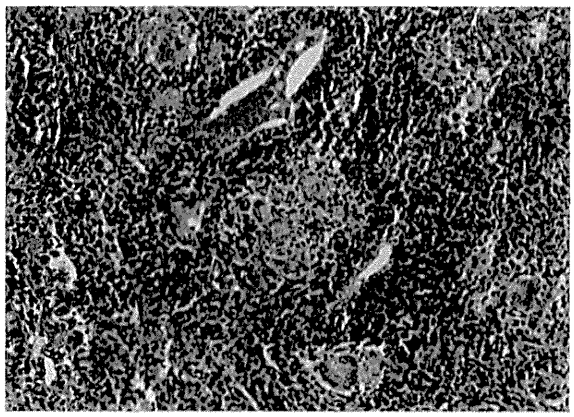

Fig. 5. Photomicrograph of biopsied lung tissue of case 2 reveals epithelioid cell granuloma with multi-nucleated giant cells and lymphocytic infiltrate (haematoxylin-eosin, × 140).

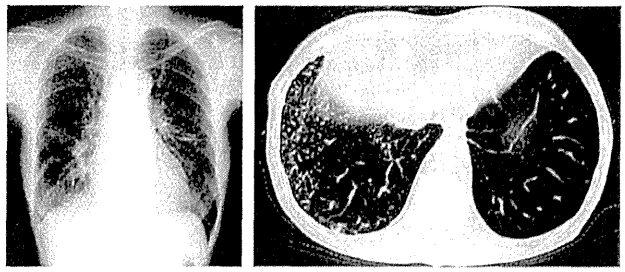

Fig. 6. Chest X-ray and CT of August 2012. The infiltrative shadow of the right lower and left upper lobes noted in Fig. 4 have improved.

and the differential diagnosis between these two diseases is particularly important. A diagnosis of sarcoidosis requires 2 or more of the following 6 findings indicating a systemic reaction: bilateral pulmonary hilar lymphadenopathy, elevated serum ACE levels, a negative tuberculin reaction, marked uptake on gallium^{67} citrate scintigraphy, lymphocytosis or an increased CD4/CD8 ratio in BAL fluid, and serum hypercalcaemia. Evaluation of these 6 items is important to exclude a diagnosis of sarcoidosis [7,8].

In patient 1 at the time of hospital admission, a QuantiFERON-TB blood assay was negative, acid-fast smear cultures of the bronchial lavage fluid were negative, and acid-fast staining of TBLB specimens was negative. Thus, tuberculosis was unlikely. Grocott staining, β-D glucan, and cryptococcal antigen testing of the TBLB specimens were negative, so a fungal infection was also unlikely. The only item meeting the diagnostic criteria for sarcoidosis was a negative tuberculin reaction, but because the QuantiFERON-TB test was also negative, this was thought to be of weak diagnostic significance, and sarcoidosis was ruled out. In addition, drug treatment had not been switched during follow-up, so drug-induced pneumonia was also unlikely. Based on a diagnosis of exclusion and the histopathology, the findings were consistent with CD-related pulmonary lesions.

In patient 2, the histopathologic examination revealed an epithelioid cell granuloma, multi-nucleated giant cells, and lymphocytic infiltration (Fig. 5). Acid-fast cultures of the bronchial lavage fluid and lung biopsy tissue were negative, so mycobacterial infection was unlikely. Sarcoidosis was also ruled out based on lack of elevation of serum ACE (15.5 IU/L) and a positive tuberculin reaction. Drug-induced lung disorder was also unlikely because the drug regimen had not been changed during outpatient treatment.

Three cases of granulomatous lung lesions in CD have been reported outside of Japan (a 13-year-old girl, a 14-year-old girl, and a 17-year-old boy). However, such cases have not been previously reported in Japan, so the present patients represent two rare cases. All 3 patients reported outside of Japan were young, had severe symptoms, and were being treated with infliximab (anti-TNF-α antibody drug). Regarding the present two cases in Japan, patient 1 (43-year-old man) was diagnosed with CD at age 11 years, and because of active gastrointestinal symptoms, infliximab (anti-TNF-α antibody drug) was started. Patient 2 (76-year-old woman) was diagnosed with CD at age 44 years, and she had a relatively satisfactory clinical course on nutritional therapy alone. If we consider the present 2 patients and the 3 patients reported from outside of Japan, 4 of these 5 patients developed CD at a young age and had highly active gastrointestinal symptoms [9]. Therefore, when lung lesions are seen in patients who developed CD during their youth and have highly active gastrointestinal symptoms, CD-related granulomatous lung lesions must be considered.

Conflict of interest

None.

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The thoracic cage becomes flattened in the progression of pleuroparenchymal fibroelastosis

To the Editor:

Pleuroparenchymal fibroelastosis (PPFE) was first reported by FRANKEL *et al.* [1]. PPFE can occur without any aetiology or underlying diseases (idiopathic PPFE), or with underlying diseases or conditions. Idiopathic PPFE has been listed as one of the rare idiopathic interstitial pneumonias (IIPs) in the revised international multidisciplinary consensus classification of IIPs [2]. The natural history of PPFE is variable, some are slowly progressive and others sometimes show rapid deterioration resulting in poor prognosis, like idiopathic pulmonary fibrosis (IPF). Idiopathic pulmonary upper lobe fibrosis (PULF), first proposed by AMITANI *et al.* [3], is currently considered to be almost identical to idiopathic PPFE [1, 4, 5], which is now globally accepted as a representative nomenclature for this disorder. Therefore, we use the term PPFE to describe the same disease as PULF.

AMITANI *et al.* [3] recognised a characteristic constitution in patients with PPFE: they are slender and their thoracic cage is flattened, *i.e.* the ratio of the anteroposterior diameter of the thoracic cage (APDT) to the transverse diameter of the thoracic cage (TDT) is abnormally lower than in normal populations. Herein, we have provisionally named this deformity of the thoracic cage as "flat chest". Other investigators have also noticed this deformity in idiopathic PPFE [6–8]. Flat chest may result from a congenital disposition or may be an acquired deformity of the thoracic cage associated with fibrosing upper lung lobes.

We assessed the APDT/TDT ratio in patients with PPFE. Subsequently, we examined whether flat chest became more pronounced during the progression of the disease. Based on clinical files from our hospitals, eight patients with PPFE in whom the APDT/TDT ratio had been measured twice with an interval of $\geqslant$1.87 years using chest computed tomography (CT) were retrospectively enrolled. TDT was determined as the longest transverse diameter of the thoracic cage measured parallel to a line (line #) along the rearmost points of the 6th thoracic vertebra in the horizontal section of the chest CT (fig. 1a). APDT was determined as the longest distance of the anteroposterior dimension of the thoracic cage measured perpendicular to the line along the rearmost points of the 6th thoracic vertebra (line #). In addition, TDT and APDT were also defined as the distances from the inside of a rib to the inside of the opposite rib. If APDT in one hemithorax was different to the APDT in the other hemithorax, a mean value was adopted. We also compared the change in forced vital capacity (FVC) during the interval between the two measurements of APDT/TDT. The first FVC was defined as the FVC measured around the first measurement of APDT/TDT, with a time lag <4% of the interval between the two measurements of APDT/TDT. The second FVC was defined as the FVC measured around the second APDT/TDT, with intervals of 1.42–4.67 years between the two measurements of FVC.

In case 2, flat chest became more pronounced after an interval of 5.27 years: APDT/TDT decreased from 0.592 to 0.509 and FVC decreased from 3540 to 1120 mL (fig. 1). In eight patients, the APDT/TDT became significantly lower after intervals of 1.87 to 5.27 years (paired t-test, p=0.003), during which FVC was remarkably decreased in six patients (table 1).

Barrel chest is a well-known acquired deformity of the thoracic cage in patients with advanced pulmonary emphysema. It is possible that a long-standing increase in intrathoracic pressure, caused by overinflated lungs associated with obstructive ventilatory impairment, leads to an expansion of the thoracic cage into

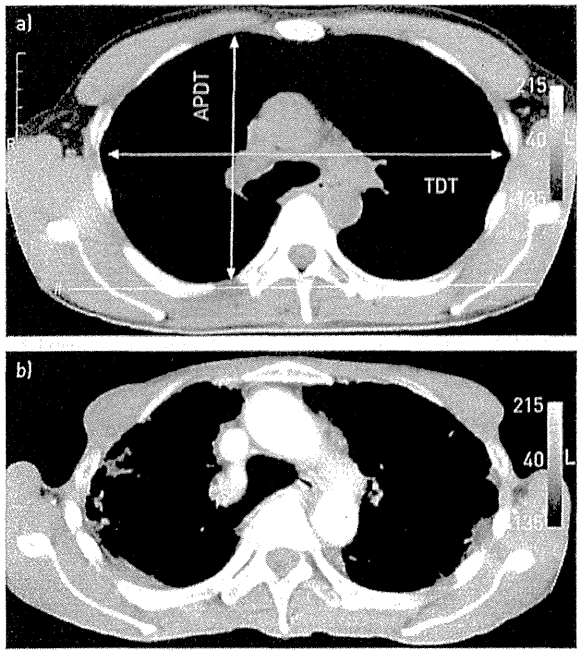

FIGURE 3 Chest computed tomography scan from a 55-year-old male with pleuroparenchymal fibroelastosis (case 2) at the level of the 6th thoracic vertebra a) on admission and b) 5.27 years later. The anteroposterior diameter of the thoracic cage (APDT)/transverse diameter of the thoracic cage (TDT) ratio decreased after 5.27 years. Note the space present between the anterior edge of the ascending aorta and the anterior chest wall in (a). The anterior edge of the ascending aorta was attached to the anterior chest wall after 5.27 years. TDT was determined as the longest transverse diameter of the thoracic cage measured parallel to a line (#) that runs along the rearmost points of the bilateral 6th ribs in the horizontal section of the CT.

barrel chest. In contrast, we have demonstrated that a long-standing fibrosing process in the bilateral upper lobes led to the contraction of the thoracic cage to flat chest in patients with PPFE. However, the wide variety of the observation periods, due to the retrospective design, is a limitation of this study.

To compare the severity of flat chest in the same condition, the APDT/TDT was measured at the level of the 6th thoracic vertebra, which includes the fibrosing bilateral upper lobes. In case 1, however, the APDT/TDT did not decrease after an interval of 4.39 years. Because the patient's flat chest was already very advanced at the time of the first measurement, there seemed to be no further apparent progression of the deformity thereafter.

AMITANI *et al.* [3] speculated that the deformity of the thoracic cage in idiopathic PPFE results from a congenital disposition. However, we found that the thoracic cage became flattened in the deteriorated course of PPFE, although the possibility that flat chest was already present before the onset of PPFE cannot be excluded.

NAKASONE *et al.* [9] reported two PPFE patients who had undergone allogenic stem cell transplantation; the thoracic cage was flattened in one of the patients. We have shown that three of the eight patients with flat chest had various underlying diseases, such as ulcerative colitis, post-irradiation therapy and lung transplantation. Such findings may support the hypothesis that flat chest is not caused by a hereditary disposition; rather, it seems to be an acquired deformity that is secondary to the progressively decreasing volume of fibrosing upper lobes. However, it is also possible that flattened chest caused by fibrosis results in immobilisation of the upper lungs which further advances fibrosis.

In PPFE, both FVC and total lung capacity are decreased, whereas the ratio of forced expiratory volume in 1 s/FVC is increased, which is similar to IPF. However, fibrotic collapse of the upper lobes leads to the compensatory over inflation of the lower lobes. This results in an increase in the ratio of residual volume/total lung capacity [10], which is a specific functional impairment not usually seen in IPF. Reciprocal mechanical vectors produced by shrinkage of upper lobes and over inflation of lower lobes might be reflected in the specific physical finding of flat chest.

TABLE 1 Change of the thoracic cage and forced vital capacity (FVC) in patients with pleuroparenchymal fibroelastosis

Case	Age years	Sex	Idiopathic or with underlying disease	Diagnostic method	APDT/TDT ratio			FVC (% predicted)		
					1st measurement	2nd measurement	Interval between measurements	1st measurement	2nd measurement	Interval between measurements
1	41	M	Ulcerative colitis	SLB	0.45	0.458	4.39 years			
2	55	M	Idiopathic	SLB	0.592	0.509	5.27 years	3540 mL (87%)	1120 mL (29%)	4.67 years
3	60	M	Idiopathic	SLB	0.599	0.554	2.79 years	2580 mL (78%)	1040 mL (32%)	2.64 years
4	78	M	Radiation therapy for oesophageal cancer	Autopsy	0.579	0.53	1.87 years	2480 mL (69%)	1370 mL (39%)	1.42 years
5	32	F	Lung transplantation	SLB	0.54	0.489	2.31 years	2050 mL (69%)	640 mL (20%)	2.47 years
6	48	F	Idiopathic	SLB	0.599	0.522	4.03 years	2030 mL (67%)	1380 mL (49%)	3.61 years
7	59	F	Idiopathic	SLB	0.652	0.6	4.59 years			
8	81	F	Idiopathic	Autopsy	0.729	0.612	2.04 years	1800 mL (83%)	1060 mL (51%)	1.84 years

APDT: anteroposterior diameter of the thoracic cage; TDT: transverse diameter of the thoracic cage; M: male; F: female; SLB: surgical lung biopsy.

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Flat chest may be an acquired deformity of the thoracic cage in patients with pleuroparenchymal fibroelastosis http://ow.ly/tyqfq

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Specific inhalation challenge tests for occupational asthma in Europe: a survey

To the Editor:

Asthma is described as "occupational" if it has been induced by an agent encountered in the workplace. The avoidance of further exposure to the causative agent often results in resolution of the asthma, especially if it can be achieved soon after the onset of symptoms [1]. Consequently, occupational asthma is one of the very few types of asthma that are potentially curable.

The implications of this include the importance of early recognition of occupational asthma and of accurate identification of the causative agent. A variety of methods are available to make a diagnosis [2], among which specific inhalation challenge (SIC) testing is generally considered to be the reference standard [3–5]. In this context, SIC testing is the controlled exposure of a patient, under laboratory conditions, to an agent

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Case report

Two cases of pulmonary lymphangioleiomyomatosis in postmenopausal women

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ABSTRACT

Pulmonary lymphangioleiomyomatosis is a rare disease that occurs exclusively in young women, who typically present with progressive dyspnea and repeated spontaneous pneumothorax. The diagnosis of this disease in elderly women with a non-progressive clinical course is extremely rare. We herein report two incidentally discovered cases of pulmonary lymphangioleiomyomatosis in postmenopausal patients. These patients had not presented with any respiratory symptoms. Lymphangioleiomyomatosis may be related to hormonal influences; however, the etiology and pathogenesis of the pulmonary lesions that develop in postmenopausal females are unclear, even after a review of the literature.

1. Introduction

Pulmonary lymphangioleiomyomatosis (LAM) is a rare pulmonary disorder characterized by multiple pulmonary cysts with abnormal proliferation in the lymphatic system. The common clinical manifestations of pulmonary LAM include progressive dyspnea on effort, repeated spontaneous pneumothorax and chylothorax [1]. This disease is extremely rare in postmenopausal patients, because it occurs almost exclusively in women of child-bearing age and is considered to develop as a result of hormonal influences.

We herein describe two cases of pulmonary LAM that were discovered incidentally in postmenopausal patients, who did not present with any respiratory symptoms at the time of diagnosis.

2. Case presentation

2.1. Case 1

A 50-year-old Japanese, non-smoking woman without any respiratory symptoms was admitted because of multiple lung cysts and a small nodule in the right lower lobe (Fig. 1A), which had been discovered on chest computed tomography (CT) performed for a general check-up before treatment of a gastric submucosal tumor. At age 32, the patient underwent a hysterectomy for treatment of myoma uteri and hence reached menopause, but was never administered estrogen replacement therapy. She had never smoked or inhaled any toxic materials. Physical examinations revealed no abnormal

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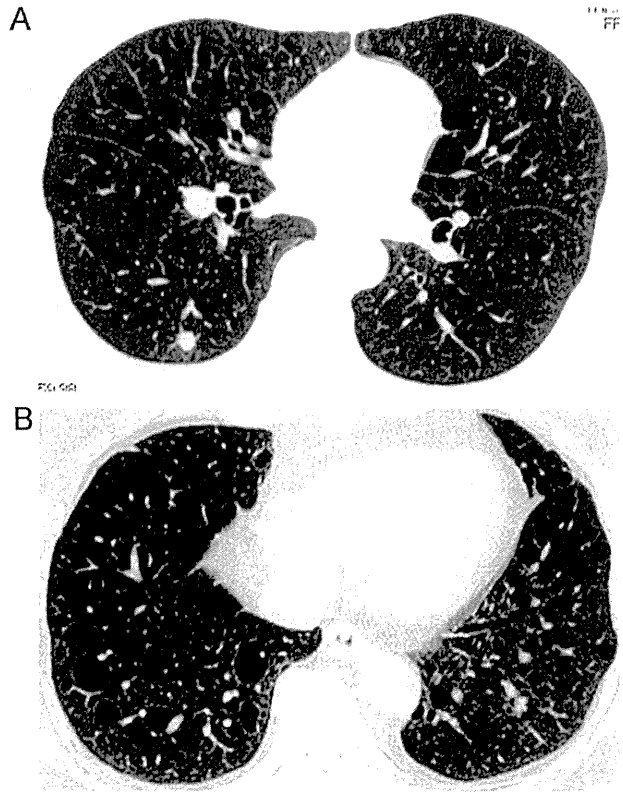

Fig. 1 – Chest computed tomography in cases 1 (A) and 2 (B), showing a small nodule in the right lower lobe (A) and bilateral multiple thin-walled cysts (A, B).

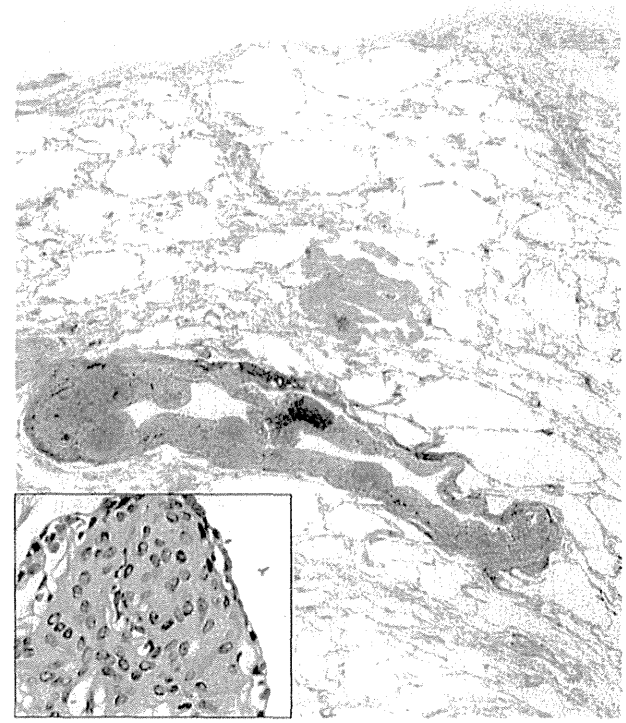

Fig. 2 – The microscopic findings related to the surgically biopsied specimen in case 1 (hematoxylin and eosin stain) showing some cyst-like spaces, mildly thickened alveolar walls, and nodular lesions. Focal proliferations of short spindle-shaped cells were present (inset).

findings of the skin; chest auscultation and SpO_2 measurements were also normal. The laboratory findings showed no abnormal data, and no α1-antitripsin deficiency was found. No abnormal findings were detected on chest radiography. Pulmonary function tests revealed a vital capacity (VC) of 2.94 L (106.5% predicted), a forced expiratory volume in one second ($FEV_{1.0}$) of 2.17 L, an $FEV_{1.0\%}$ of 81.9%, and a percent diffusion lung capacity for carbon monoxide ($\%D_{LCO}$) of 71.3%. A surgical lung biopsy was performed, and the small nodule in the right lower lobe was diagnosed as an intrapulmonary lymph node.

Immunohistochemical staining of the lung specimen (Fig. 2) was positive for α-smooth muscle actin, HMB45, the estrogen receptor, and the progesterone receptor (data not shown). Therefore, the patient was diagnosed with pulmonary LAM. Findings of tuberous sclerosis were not observed in other organs. Thus far, we have not been able to obtain permission from the patient for tumor suppressor gene (tuberous sclerosis complex; TSC) testing. In the 3 years that have elapsed since the patient's diagnosis, pulmonary function tests and chest CT findings have shown no deterioration.

2.2. *Case 2*

A 66-year-old Japanese woman without any respiratory symptoms was referred because of multiple lung cysts (Fig. 1B) that had been discovered on chest CT performed for a general check-up before surgery for dermatofibrosarcoma protuberans of the neck. The patient had reached menopause at age 52, and was never prescribed estrogen replacement therapy. At age 58, she underwent hysterectomy and oophorectomy for treatment of uterine cancer with simultaneous resection of a renal tumor. The resected tissue was pathologically diagnosed as renal angiomyolipoma. She had never smoked or inhaled any toxic materials. No past or family history of spontaneous pneumothorax was reported. Physical and laboratory examinations showed normal results. The patient's chest radiography showed normal results. Pulmonary function tests revealed a VC of 1.92 L (83.8% predicted), $FEV_{1.0}$ of 1.30 L, $FEV_{1.0\%}$ of 75.1%, and $\%D_{LCO}$ of 62.0%. No findings of tuberous sclerosis were observed. Unfortunately, we have not been able to obtain permission from the patient or family for TSC genetic testing. Although no lung biopsy was performed for confirmation, the patient did not show characteristics of other diseases that can cause pulmonary cysts, such as pulmonary emphysema, Langerhans' cell histiocytosis, and Sjogren's syndrome. The patient met the diagnostic criteria [2] for LAM with renal angiomyolipoma. There has been no deterioration detected by pulmonary function tests or chest CT findings during the 2 years since the diagnosis.

3. Discussion

We herein presented two cases of LAM that developed after gynecological surgery in postmenopausal women who had not taken any estrogen hormone replacement therapy.

Neither of the patients had progressive dyspnea nor repeated spontaneous pneumothorax, and there was a possibility that the onset of LAM had occurred after menopause in both cases. The previous [3-13] and present reports of 15 postmenopausal patients with pulmonary LAM (mean age of onset; 62.7 years) (Table 1) showed that eight patients underwent gynecological surgery (hysterectomy and/or oophorectomy, salpingectomy), while the others experienced natural menopause. Five of the 15 patients had received estrogen hormone replacement therapy after menopause (Cases 5, 6, 8, 9, and 12). Three patients were treated for LAM with progesterone or tamoxifen (Cases 7-9). When compared to the patients who did not receive treatment, these cases exhibited slight improvements in pulmonary function and arterial oxygen tension and had prolonged survival. In contrast, six patients, including the two cases presented here, remained stable in terms of CT findings and pulmonary function, without any treatment. Thus, the effects of anti-estrogenic therapy among these 15 patients are controversial.

Seyama et al. reported that five of 11 premenopausal patients with pulmonary LAM who had not received anti-hormone therapy showed slower progression of the cystic changes on CT and pulmonary function tests, which was not the case for the six patients who had received anti-hormone therapy [14]. Furthermore, one large study of patients with LAM showed no benefit of treatment in terms of the decline in lung function [15]. Therefore, we think that anti-estrogenic therapy may not always be necessary for LAM patients, especially those in whom disease onset occurs after menopause, and who exhibit a stable course without any hormone-related therapy.

The etiology of LAM is still unclear. LAM cells in the lungs are immunohistochemically positive for estrogen and progesterone receptors, and usually are limited to the large epithelioid cells, but there is no evidence that individuals with estrogen receptor-positive pulmonary LAM tend to respond to anti-estrogenic therapy. In most patients with pulmonary LAM, the LAM cells contain somatic mutations of the TSCs. The *TSC1* gene is located at 9q34 and encodes the hamartin protein, while *TSC2* is located at 16p13.3 and encodes tuberin [16]. TSC1 and TSC2 form a heterodimer complex, where hamartin is the regulatory component stabilizing tuberin. Deficiencies or dysfunction of either hamartin or tuberin activate the mammalian target of rapamycin (mTOR) and other downstream proteins, resulting in increased protein translation and inappropriate cellular proliferation [17]. The *TSC1*/*TSC2*-related signaling pathways are also involved in the pathogenesis of pulmonary LAM. Currently, it is thought that both sporadic pulmonary LAM and pulmonary LAM associated with the tuberous sclerosis complex occur via a two-hit mechanism with a mutation of either the *TSC2* or *TSC1* gene, followed by a second hit leading to a loss of heterozygosity, causing the loss of function of either protein. However, no model of pulmonary LAM cells is available thus far, and the relative contributions of *TSC1*/*TSC2* have not been clarified. On the other hand, sporadic cases of pulmonary LAM show a TSC2 mutation predominance, and it was reported that the size of lung cysts did not differ in tuberous sclerosis patients with either the *TSC1* or *TSC2* mutation, while patients with *TSC2* mutations had more cysts than patients with *TSC1* mutations [18]. Another study [19] demonstrated that tuberous sclerosis patients with *TSC1* mutations had, on average, milder disease than those with *TSC2* mutations. Linking the distribution and types of *TSC1* or *TSC2* mutations with a specific gene region may affect treatment decisions and prognostic determinations. Considering these facts and the instances of LAM observed in postmenopausal women, we believe that excess estrogen and progesterone deficiency are not the primary causes of the disease, but may be one of the triggers for pulmonary LAM progression. Recent therapeutic trials with sirolimus, an inhibitor of mTOR, have shown stabilized lung function associated with a reduction in symptoms and improvement in the quality of life [20]. However, the indications for sirolimus treatment for LAM in postmenopausal women currently remain unknown.

Further studies on the role of TSC are needed to elucidate the relationships with the age of onset and/or the degree of LAM progression. Clinicians should be aware of the potential for pulmonary LAM in the differential diagnosis of pulmonary

Table 1 – Reported postmenopausal cases of pulmonary lymphangioleiomyomatosis.

	Year	Age	History of surgery	Estrogen replacement	Symptom	Treatment	Outcome
1 [3]	1964	69	(−)	(−)	Dyspnea	(−)	N.D.
2 [4]	1973	65	Salpingectomy	(−)	Dyspnea	(−)	Died
3 [5]	1980	70	(−)	(−)	Dyspnea	(−)	Survived
4 [6]	1985	72	Hysterectomy	(−)	Dyspnea	(−)	Died
5 [7]	1990	49	Hysterectomy, oophorectomy	(+)	N.D.	N.D.	N.D.
6 [7]		61	Hysterectomy, oophorectomy	(+)	N.D.	N.D.	N.D.
7 [8]	1994	59	(−)	(−)	Dyspnea	Tamoxifen	Survived
8 [8]		62	Hysterectomy, oophorectomy	(+)	Dyspnea	Progesterone	Survived
9 [9]	1996	62	(−)	(+)	Dyspnea	Progesterone	Survived
10 [10]	2001	75	(−)	(−)	(−)	(−)	Unknown
11 [11]	2007	59	(−)	(−)	(−)	N.D.	N.D.
12[12]	2009	51	Hysterectomy, oophorectomy	(+)	(−)	(−)	Survived
13 [13]	2010	70	(−)	(−)	Dyspnea	N.D.	N.D.
Present cases		50	Hysterectomy	(−)	(−)	(−)	Survived
		66	Hysterectomy, oophorectomy	(−)	(−)	(−)	Survived

N.D.: not described.

cystic diseases, and should consider LAM even in elderly patients. More postmenopausal cases of LAM should be reviewed to determine the proper treatment and help elucidate the pathogenesis of the disease.

Conflict of interest

The authors have no conflicts of interest.

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