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Effects of molecular structural variants on serum Krebs von den Lungen-6 levels in sarcoidosis

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Abstract

Background: Serum Krebs von den Lungen-6 (KL-6), which is classified as human mucin-1 (MUC1), is used as a marker of sarcoidosis and other interstitial lung diseases. However, there remain some limitations due to a lack of information on the factors contributing to increased levels of serum KL-6. This study was designed to investigate the factors contributing to increased levels of serum KL-6 by molecular analysis.

Methods: Western blot analysis using anti-KL-6 antibody was performed simultaneously on the bronchoalveolar lavage fluid (BALF) and serum obtained from 128 subjects with sarcoidosis.

Results: KL-6/MUC1 in BALF showed three bands and five band patterns. These band patterns were associated with the *MUC1* genotype and the KL-6 levels. KL-6/MUC1 band patterns in serum were dependent on molecular size class in BALF. Significantly increased levels of serum KL-6, serum/BALF KL-6 ratio and serum soluble interleukin 2 receptor were observed in the subjects with influx of high molecular size KL-6/MUC1 from the alveoli to blood circulation. The multivariate linear regression analysis involving potentially relevant variables such as age, gender, smoking status, lung parenchymal involvement based on radiographical stage and molecular size of KL-6/MUC1 in serum showed that the molecular size of KL-6/MUC1 in serum was significant independent determinant of serum KL-6 levels.

Conclusions: The molecular structural variants of KL-6/MUC1 and its leakage behavior affect serum levels of KL-6 in sarcoidosis. This information may assist in the interpretation of serum KL-6 levels in sarcoidosis.

Keywords: Serum KL-6, Molecular structural variant, Sarcoidosis

Background

Krebs von den Lungen-6 (KL-6) is a mucinous sialylated sugar chain on human mucin-1 (MUC1) [1,2]. MUC1 consists of a large extracellular domain, a single-pass transmembrane region, and an intracellular cytoplasmic tail [3,4]. The large extracellular domain contains a variable number of tandem repeat (VNTR) regions that are heavily glycosylated (Figure 1). In normal lung tissue, KL-6 is expressed on type II pneumocytes [1,5]. KL-6 is present in high concentrations in bronchoalveolar lavage fluid (BALF) and also circulates in blood [6]. Serum KL-6 is specifically elevated in a majority of patients with interstitial lung diseases (ILDs), and this phenomenon is considered to reflect the production by regenerating type II epithelial cells based on disease activity [6-13]. Therefore, measurement of serum KL-6 is widely accepted, particularly in Japan, as a diagnostic test for ILDs and a marker of disease activity.

Sarcoidosis is a multiorgan inflammatory disease of unknown origin that is characterized by noncaseating epithelioid cell granuloma and lymphocytic alveolitis [14]. Because the natural history and prognosis of sarcoidosis are unpredictable, it is important to monitor disease development during management [14]. KL-6 is considered to be one of the useful serum markers for monitoring diseases activity in sarcoidsis. Several investigators have reported that levels of serum KL-6 reflect lymphocytic alveolitis and increased parenchymal infiltration [11-13]. However, we have experienced some limitations in the

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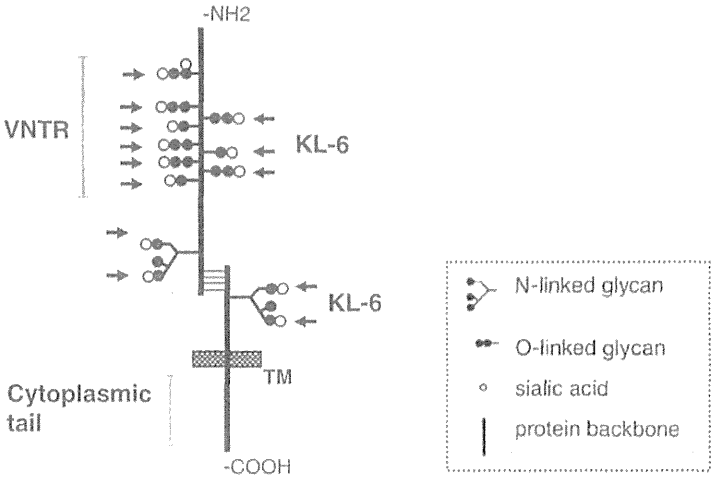

Figure 1 Possible diagrammatic structure of MUC1 showing KL-6 epitopes. TM, transmembrane; VNTR, variable number of tandem repeats. Arrows indicate KL-6 antigens on MUC1 protein.

interpretation of serum KL-6 levels, which include its dissociation with disease activity and different behavior from other serum markers in some cases. This prompted us to examine the factors contributing to increased levels of serum KL-6.

There are known variations in the length and structure of the MUC1 protein that result from polymorphisms in the VNTR [15,16]. The size class of MUC1 protein in tears is reported to be linked with the genotype of a single nucleotide polymorphism (SNP) in exon 2 (rs4072037) of the *MUC1* gene [16]. In addition, Janssen et al. recently reported an association between this polymorphism and variations in serum KL-6 levels in healthy individuals and patients with pulmonary sarcoidosis [17]. Based on these reports, we hypothesized that: 1) various molecular sizes of KL-6/MUC1, which are genetically determined by *MUC1* gene polymorphism (such as rs4072037), would be present in BALF; 2) the influx of KL-6/MUC1 from alveoli to blood is dependent on the molecular size of KL-6/MUC1; and finally, 3) serum KL-6 levels would be affected by the molecular size and leakage behavior of KL-6/MUC1, in addition to increased local production of KL-6/MUC1 in lung.

In this study, we examined the factors contributing to the variable increases in serum levels of KL-6 using molecular analysis in patients with sarcoidosis, all of whom simultaneously underwent blood sampling and bronchoalveolar lavage (BAL).

Materials and methods

Subjects

A total of 128 subjects with pulmonary sarcoidosis visiting the pulmonary clinic of the First Department of Medicine, Hokkaido University Hospital between April 2000 and July 2011 were enrolled into this study. The diagnosis of pulmonary sarcoidosis was established based on clinical findings and histologic evidence of noncaseating epithelioid cell granulomas, after excluding known causes of granulomatous diseases, in accordance with the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders guidelines [14]. All subjects underwent BAL, which is a routine diagnostic procedure at our hospital for patients with undiagnosed sarcoidosis, as described previously [18,19]. Serum samples were collected 30 minutes before BAL in all subjects. The study population, sex, age, smoking history, radiographical stage [14], BALF findings, pulmonary functions results, and levels of serum markers are shown in Table 1. The smoking status of one subject was unknown. BALF cell analysis of 1 patient was not performed due to problems with BAL storage. Pulmonary function data were available from 122 patients.

All patients had provided written informed consent for their samples to be used in future clinical research [18,19]. The Institutional Review Board of Hokkaido University Hospital for Clinical Research approved the study protocols (approval No. 009–0295).

Western Blotting

Western blotting was performed on BALF and serum from all subjects. Briefly, protein samples from BALF and serum were electrophoresed on 3%–8% NUPAGE Tris-acetate gels (Invitrogen, Carlsbad, CA) and were transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with PBS containing 3% skim milk. Western blot analysis was performed using anti-KL-6 antibody (anti-KL-6 antibody was kindly provided

Table 1 Characteristics of the study population

	Sarcoidosis
No. of subjects	128
Men/Women	34/94
Age, yr	55(17–79)
Cigarette smoking (never/former/current)	55/29/43
Radiograhical stage (0/I/II/III)	21/56/43/8
BALF findings	
Total cell counts, 10^4/mL	18.1 (3.2–92.2)
Macrophages, %	66.3 (6.0–97.0)
Lymphocytes, %	32.8 (2.9–76.3)
CD4/CD8, ratio	4.93 (0.65–30.16)
Pulmonary function result	
VC, % predicted	113.0 (70.5–153.7)
D_{LCO}, % predicted	90.1 (37.0–142.0)
Serum maker	
soluble IL-2 receptor, U/mL	805 (117–4990)
KL-6 levels	
BALF, U/mL	296 (90–1507)
serum, U/mL	336 (102–3091)

Data are presented as median (range).

by Sanko Junyaku Co., Ltd.) and DF-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by alkaline phosphatase-conjugated goat anti-mouse Ig. Bands were developed using the WesternBreeze Chromogenic Immunodetection Kit (Invitrogen).

Genotyping of *MUC1* polymorphism

The *MUC1* SNP (exon 2; rs4072037) was genotyped in 80 patients with sarcoidosis, who had consented to future genetic studies, using the TaqMan system (Assay ID: C_27532642_10, Applied Biosystems, Foster City, CA).

Measurement of KL-6, albumin and soluble interleukin 2 receptor

KL-6 levels in both BALF and serum were measured by electrochemiluminescent immunoassay using the PICOLUMI KL-6 kit (Sanko Junyaku, Tokyo, Japan). Reference intervals were 105.3–401.2 U/mL for Japanese normal subjects. Albumin levels in BALF were measured on a Hitachi 7070 automated analyzer with TAC-2 test Albumin U (Medical and biological laboratories co., LTD., Nagoya, Japan). Serum soluble interleukin 2 (IL-2) receptor was measured by a solid-phase, two-site chemiluminescent immunometric assay (IMMULITE 2000 IL2R, Siemens Healthcare Diagnostics, Los Angeles, CA).

Statistical methods

Statistical analysis was performed with SYSTAT 11 for Windows (Systat Inc., Chicago, IL) and SAS (SAS Institute, Inc., Cary, NC). Data were expressed as median and ranges. All data were not normally distributed on univariate analysis, the natural logarithm of all data were used for further statistical analyses. Comparisons were performed by unpaired *t*-test or ANOVA adjusting potentially relevant variable such as cigarette smoking when assessing the influence of smoking status (never, ex or current). Differences between groups were evaluated by ANOVA and were assessed by Bonferroni post-hoc test. The relationship between leakage behavior of KL-6/MUC1 and smoking status was assessed using χ^2-test. Correlations between different parameters were determined by Pearson's correlation coefficient. We used Haploview software version 4.1 (http://www.broad.mit.edu/mpg/haploview; Barrett et al. 2005) in order to compare the observed numbers of genotypes with the number of expected genotypes under Hardy-Weinberg equilibrium using χ^2-test. The association between *MUC1* genotypes and KL-6/MUC1 band patterns in BALF was assessed using Cochran-Mantel-Haenszel Statistics. In order to identify independent factors predictive for the serum KL-6 levels, the multivariate linear regression analysis involving potentially relevant variables was performed. A p value of <0.05 was regarded as significant.

Results

Western blot analysis

Western blot analysis of BALF with anti-KL-6 antibody revealed three bands (low molecular size (L), middle molecular size (M) and high molecular size (H), at approximately 400, 450 and 500 kDa, respectively) under reducing conditions. Furthermore, based on the combination of these bands, five band patterns (L alone, L/M, L/H, M/H and H alone) were identified (Figure 2A). In addition, Western blot analysis with DF-3 antibody showed the same band patterns corresponding to the KL-6/MUC1 band patterns in BALF (Figure 3). All Subjects who displayed the L alone pattern in BALF also displayed L alone bands in serum (Figure 2B; Sar 1). On the other hand, subjects who displayed non-L alone patterns in BALF showed diverse patterns in serum. Based on the similarities and differences in band patterns between BALF and serum, we classified these subjects into two groups; "non-leakage" and "leakage". The non-leakage group displayed only L bands or neither M nor H bands in serum, despite the presence of M or H bands in BALF (Figure 2B; Sar 2, 3 and 4). In contrast, the leakage group displayed identical band patterns between BALF and serum (Figure 2B; Sar 5, 6 and 7). The frequency and percentage of KL-6/MUC1 band patterns in BALF and in serum are summarized in Table 2. Thirty-one of 47 subjects

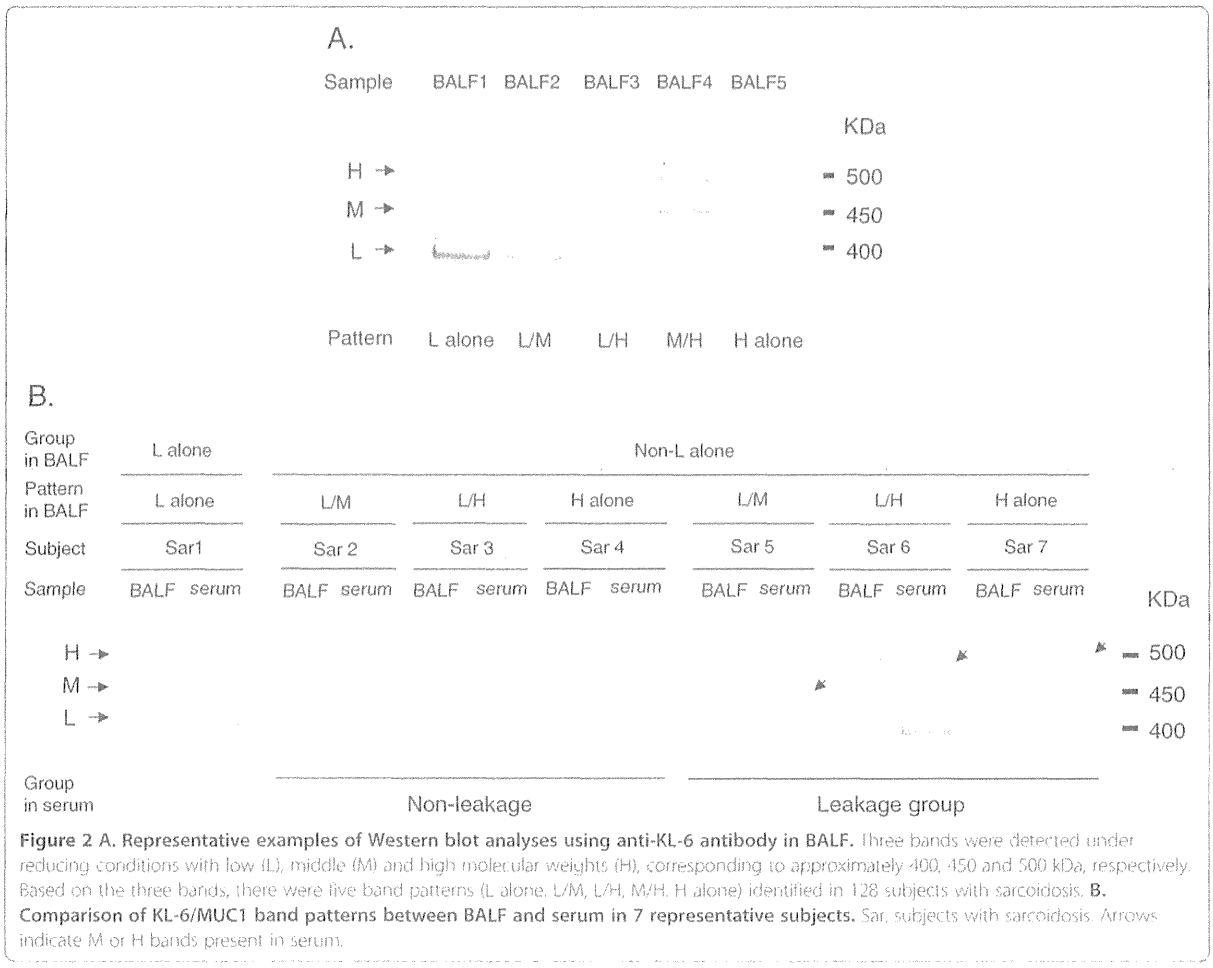

Figure 2 A. Representative examples of Western blot analyses using anti-KL-6 antibody in BALF. Three bands were detected under reducing conditions with low (L), middle (M) and high molecular weights (H), corresponding to approximately 400, 450 and 500 kDa, respectively. Based on the three bands, there were five band patterns (L alone, L/M, L/H, M/H, H alone) identified in 128 subjects with sarcoidosis. **B. Comparison of KL-6/MUC1 band patterns between BALF and serum in 7 representative subjects.** Sar, subjects with sarcoidosis. Arrows indicate M or H bands present in serum.

with non-L alone in BALF (66.0%), including 5 subjects in whom bands were not detected in serum, were classified as "non-leakage". The leakage behavior of high molecular size KL-6/MUC1 (i.e., M or H band) appeared to be influenced by smoking status (never, ex or current), but it did not reach statistical significance (χ^2-test, $p > 0.05$) (Table 3).

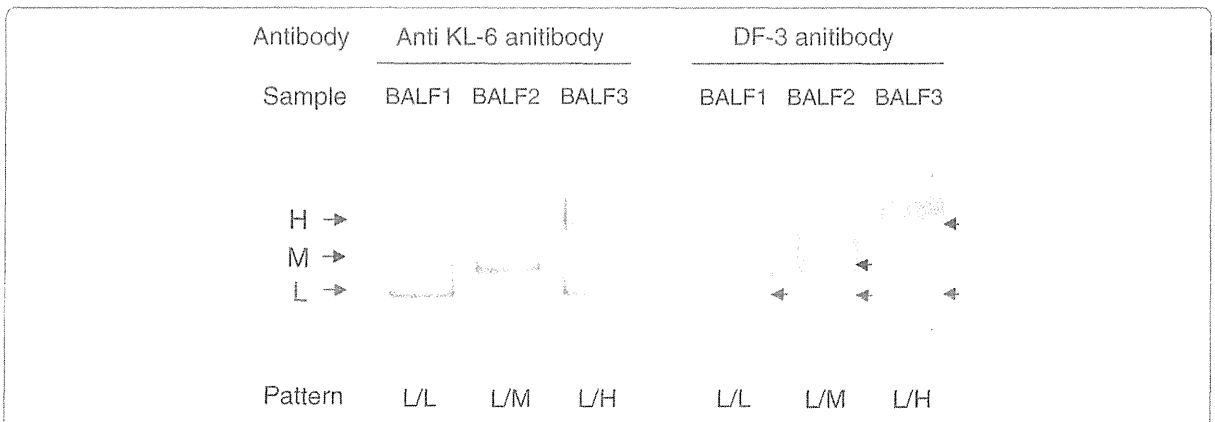

Figure 3 Representative examples of Western blot analysis using anti-KL-6 antibody and DF-3 antibody in BALF. A, anti-KL-6 antibody; B, DF-3 antibody. Arrows indicate L, M or H bands.

Table 2 The frequency and percentage of KL-6/MUC1 band patterns in between BALF and serum

			Band pattern in serum					
			L alone	L/M	L/H	H alone	Not detected	Total
Band pattern in BALF	L alone		81					81
			100.0%					100.0%
	Non L alone	L/M	8 *	8 **				16
			50.0%	50.0%				100.0%
		L/H	18 *		6 **			24
			75.0%		25.0%			100.0%
		M/H					3 *	3
							100.0%	100.0%
		H alone				2 **	2 *	4
						50.0%	50.0%	100.0%
Total			107	8	6	2	5	128
			83.6%	6.3%	4.7%	1.6%	3.9%	100.0%

*, non-leakage group; **, leakage group.

Relationship between KL-6 levels and KL-6/MUC1 size class

BALF KL-6 levels in subjects with the H alone or M/H, L/H and L/M patterns in BALF were significantly higher than those with the L alone pattern in BALF ($p < 0.001$, <0.001 and 0.003, respectively) (Figure 4A), thus suggesting that larger molecular size of KL-6/MUC1 is associated with higher levels of BALF KL-6. Similarly, serum KL-6 levels in subjects with the L/H pattern in serum were significantly higher than those with the L alone pattern ($p < 0.001$) (Figure 4B).

Relationship between *MUC1* genotypes and KL-6/MUC1 size

We examined the allele frequency of rs4072037. There was 129 (80.6%) for A and 31 (19.4%) for G. The genotype frequencies were 53 (66.3%) for AA, 23 (28.7%) for AG, and 4 (5.0%) for GG. No significant deviation from the Hardy-Weinberg equilibrium was observed ($p > 0.05$). When the KL-6 levels in both serum and BALF were grouped according to the genotype, the results were AA (serum: 285U/mL, 102–2627; BALF: 240U/mL, 90–1224), AG (serum: 480U/mL, 183–3091, BALF: 662U/mL, 130–1336) and GG (serum: 437U/mL, 337–1600; BALF: 444U/mL, 219–633) The KL-6 levels in both serum and BALF from the subjects with AG genotype were higher than those with AA genotype ($p < 0.001$, <0.001, respectively). Table 4 shows the relationship between *MUC1* genotypes and KL-6/MUC1 band patterns in BALF. There is a significant relationship between genotype and KL-6/MUC1 band patterns in BALF (Cochran-Mantel-Haenszel Statistics, $p < 0.001$); the A allele is linked with L bands and the G allele with H bands.

Table 3 Relationship between leakage behavior of high molecular size KL-6/MUC1 and smoking status

		Leakage behavior of high molecular size KL-6/MUC1		Total
		Non-leakage	Leakage	
Smoking status	Non/Ex smoker	22	8	30
		73.3%	26.7%	100.0%
	Current smoker	9	8	17
		52.9%	47.1%	100.0%
Total		31	16	47
		66.0%	34.0%	100.0%

X^2-test, $p > 0.05$.

Determination of leakage behavior of high molecular size KL-6/MUC1

As the subjects who displayed non-L alone patterns in BALF showed diverse band patterns in serum, we examined the presence of differences in the leakage behavior of high molecular size KL-6/MUC1 (i.e., M or H band). We compared the serum KL-6 levels, the serum/BALF KL-6 ratio, the BALF albumin levels, the numbers of lymphocytes and CD_4 positive cells in BALF and the serum soluble IL-2 receptor levels between the non-leakage and the leakage groups. As expected, the serum KL-6 levels in the leakage group were significantly higher than those in the non-leakage group ($p = 0.023$, Figure 5A). In addition, there was a significant increase in the serum/BALF KL-6 ratio in the leakage group, when compared with the non-leakage group ($p = 0.002$, Figure 5B). This result remained significant even after controlling for the smoking status (ANOVA, $p = 0.005$). In contrast, no significant differences in the BALF albumin levels between the non-leakage and the leakage groups were observed ($p = 0.510$, Figure 5C). The leakage group tended to have the slightly higher numbers of lymphocytes and CD_4 positive cells in BALF than the non-leakage group ($p = 0.057$ and 0.068, respectively)

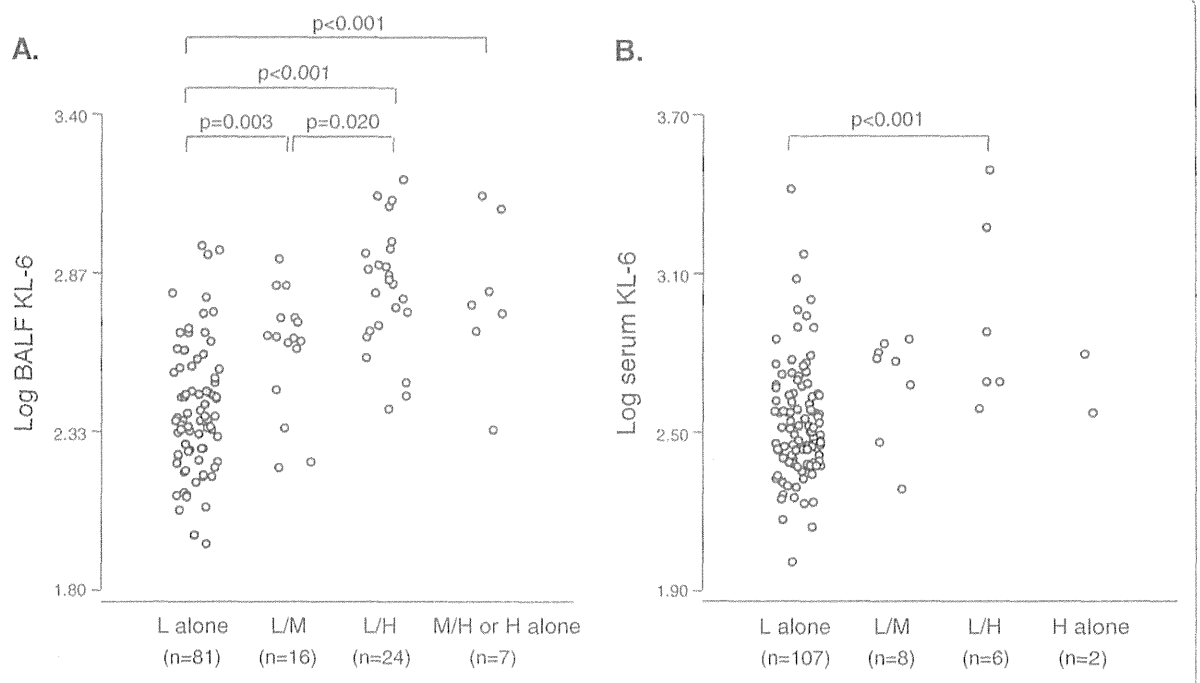

Figure 4 A. Comparison of KL-6 levels in BALF based on KL-6/MUC1 band pattern in BALF. B. Comparison of KL-6 levels in serum based on KL-6/MUC1 band pattern in serum.

(Figure 5D, E). In addition, the serum soluble IL-2 receptor levels in the leakage group was significantly higher than the non-leakage group ($p = 0.026$, Figure 5F).

Impact of KL-6/MUC1 molecular structural variants on serum KL-6 levels

Recent reports have shown that serum KL-6 levels may be affected by several potential factors, such as age, gender and smoking status, other than lung diseases [17,20]. In addition, previous reports have found that radiographical staging showing parenchymal involvement (stages II and higher) is correlated with significantly higher KL-6 levels, as compared to patients without parenchymal involvement (stages 0 and I) [10-12]. In univariate analyses between serum KL-6 levels and potentially relevant variables, there were significant differences between the subjects without and with lung parenchymal involvement based on radiographical stage [14] ($p = 0.020$) and those with low and higher molecular size KL-6/MUC1 in serum ($p < 0.001$) (Table 5). In order to identify independent factors predictive for the serum KL-6 levels, the multivariate linear regression analysis involving potentially relevant variables such as age (years), gender (male or female), smoking status (never, ex or current), lung parenchymal involvement (without or with parenchymal infiltration) and molecular size of KL-6/MUC1 in serum (low or higher molecular size) was performed. The multivariate linear regression analysis showed that only molecular size of KL-6/MUC1 in serum (Standardized β coefficient = 0.365, $p < 0.001$) was significant independent determinant of serum KL-6 levels (Table 6).

Table 4 Relationship between MUC 1 gene polymorphism (rs4072037) and KL-6/MUC 1 band patterns in BALF

		KL-6/MUC1 band pattern in BALF					
		L alone	L/M	L/H	M/H	H alone	Total
rs4072037	AA	47	5	1			53
		88.7%	9.4%	1.9%			100.0%
	AG	1	6	15	1		23
		4.3%	26.1%	65.2%	4.3%		100.0%
	GG			1	1	2	4
				25.0%	25.0%	50.0%	100.0%
Total		48	11	17	2	2	80
		60.0%	13.8	21.3%	2.5%	2.5%	100.0%

Cochran-Mantel-Haenszel Statistics, $p < 0.001$.

Discussion

The increased levels of serum KL-6 in patients with ILDs are thought to result from an increased local production of KL-6/MUC1 in lung and enhanced permeability following the destruction of the alveolar-blood interface [5,21]. In this study, we discovered three important findings with regard to the factors contributing to the variably in serum levels of KL-6. First, KL-6/MUC1 in BALF showed three bands and five band patterns, and these band patterns were associated with *MUC1* genotype and KL-6 levels. Second, the KL-6/

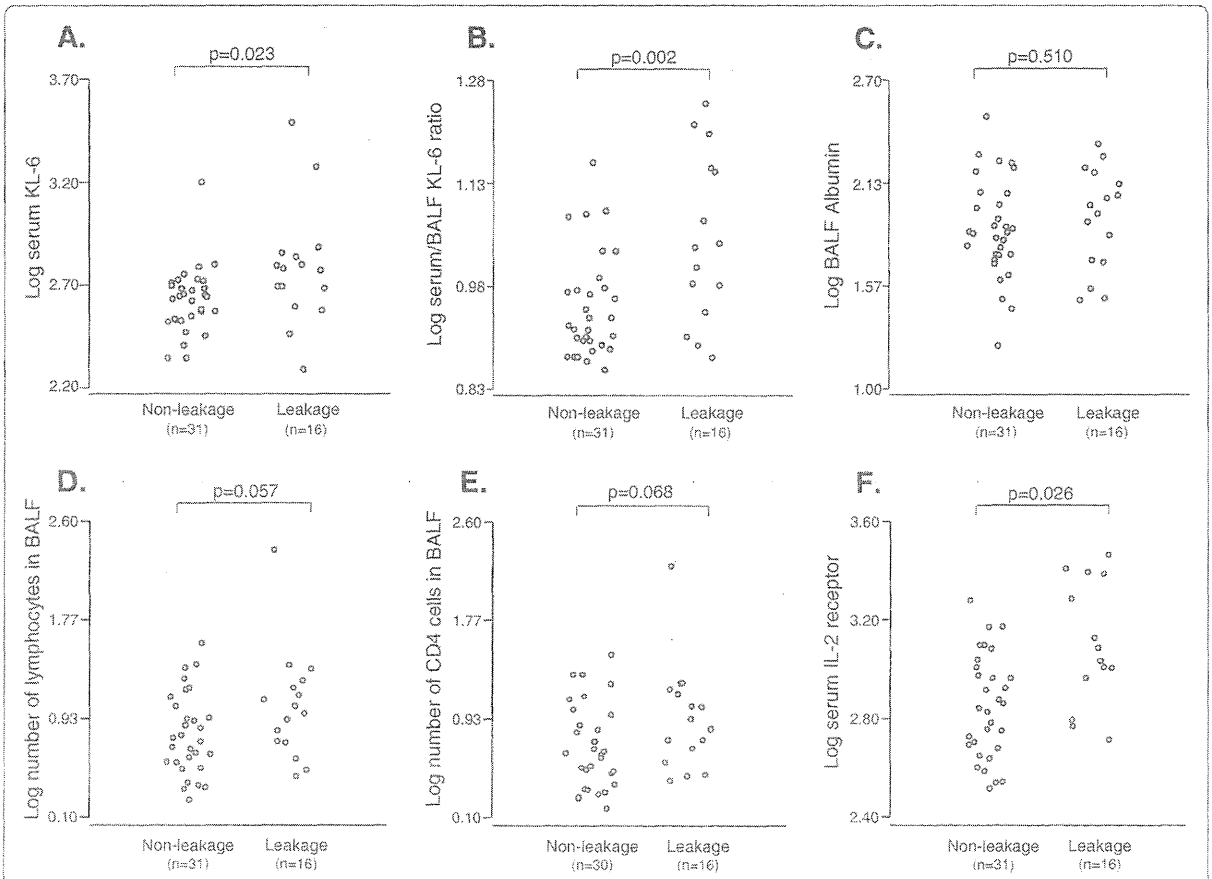

Figure 5 A, B, C, D, E and F. Comparison of serum KL-6 levels, serum/BALF KL-6 ratio, BALF albumin levels, number of lymphocytes and CD_4 positive cells in BALF and serum soluble IL-2 receptor levels between non-leakage and leakage in subjects who displayed non-L alone band pattern in BALF.

MUC1 band patterns in serum were dependent on molecular size class in BALF, as proteins of different size classes did not pass through the alveolar-blood interface in a similar manner. Finally, the molecular structural variants of KL-6/MUC1 and its leakage behavior affect serum KL-6 levels. We believe that what we found in this study would provide new insight into understanding the limitations related to the measurement of serum KL-6.

Before further discussion on these issues, we should comment on the limitations of this study. It is possible that the size classes of KL-6/MUC1 detected by Western blot analysis using anti-KL-6 antibody were affected by glycosylation of MUC1, and may not strictly reflect molecular weight. However, Western blot analysis using DF-3 antibody, which recognize the core peptide of MUC1 [22,23], displayed the same band patterns, suggesting that the glycosylation of MUC1 had little influence on the molecular size of KL-6/MUC1 characterized by Western blot analysis using anti-KL-6 antibody. We could not examine the local production of KL-6/MUC1 and quantitatively analyze each molecular size of KL-6/MUC1 in this study. The local production of KL-6/MUC1 may still be important for the interpretation of serum KL-6 levels.

The larger MUC1 proteins may express more KL-6 on its surface than the smaller MUC1 proteins. In this study, KL-6 levels in BALF, which are little influenced with alveolar-blood interface, from the subjects with larger molecular size of KL-6/MUC1 (i.e. M and H band) were significantly higher than those with low molecular size (i.e. L band). Janssen et al. reported that the *MUC1* genotype was of strong influence on serum KL-6 levels in the Caucasian population and also speculated that the positive association between *MUC1* gene polymorphism and serum KL-6 levels is caused by *MUC1* allele-related molecular size. The *MUC1* genotype was clearly linked with KL-6/MUC1 band pattern and related to KL-6 levels in our study. Therefore, KL-6 levels are affected by genetically determined molecular sizes of KL-6/MUC1. Furthermore, our results showed that significantly

Table 5 Univariate analyses between Log serum KL-6 and potentially relevant variables

		Correlation with Log serum KL-6	
		R*	*P*-value
Age		0.065	0.466
		Mean ± SD	*P*-value**
Gender	Male	2.57 ± 0.237	0.726
	Female	2.56 ± 0.264	
Smoking status	Non/Ex smoker	2.54 ± 0.205	0.072
	Current smoker	2.62 ± 0.302	
Lung parenchymal involvement	Without	2.53 ± 0.198	0.020
	With	2.63 ± 0.291	
Band pattern in serum	Low molecular size	2.54 ± 0.223	<0.001
	Higher molecular size	2.78 ± 0.284	

* Pearson correlation coefficient; ** unpaired *t*-test.

increased levels of serum KL-6 were observed in the subjects with influx of high molecular size KL-6/MUC1 from the alveoli to blood circulation. This is the first reports describing the influence of leakage behavior of high molecular size KL-6/MUC1 on serum KL-6 levels. Considering for the molecular size of KL-6/MUC1 and its leakage behavior may increase the value of serum KL-6 as a marker of sarcoidosis.

With regard to *MUC1* gene polymorphism and KL-6/MUC1 band patterns in BALF, although the A allele was linked with the L band and the G allele was linked with the H band, 5 of 53 subjects (9.4%) with the AA genotype, 7 of 23 subjects (30.4%) with the AG genotype and 1 of 4 subjects (25.0%) with the GG genotype exhibited the M band. *MUC1* alleles can mostly be divided into size classes containing small (30–45) or large (60–90) numbers of repeats on Southern blot analysis [15,24]. In other words, the molecular size of the protein, which directly reflects the allele length, is associated with the number of tandem repeats on VNTR regions. We thus speculate that the presence of M bands in BALF indicates the presence of intermediate numbers of tandem repeats in VNTR regions.

Table 6 Independent factors predictive for serum KL-6 levels

	Standardized β coefficient	*P*-value
Age	0.040	0.673
Gender	−0.025	0.782
Smoking status	0.087	0.353
Lung Parenchymal involvement*	0.125	0.164
Molecular size of KL-6/MUC1 in serum	0.365	<0.001

* Based on radiographical stage.

Influx mechanism of KL-6/MUC1 from the alveoli to blood circulation is unclear. In sarcoidosis, increased levels of BALF albumin are thought to result from an influx of plasma albumin into the alveoli [25]. To analyze the leakage behavior of high molecular size KL-6/MUC1, we also measured the concentration of BALF albumin in subjects with non-L alone pattern in BALF. No significant differences between the groups of non-leakage and leakage of high molecular size KL-6/MUC1 were observed in the BALF albumin levels. In contrast, there was a significant increase in the serum/BALF KL-6 ratio, which is an indicator of leakage behavior, in the leakage group. The influx of the high molecular size KL-6/MUC1 may be not parallel to albumin-size proteins and be complicated.

The passage of high molecular size KL-6/MUC1 from alveoli to blood may be regulated by pore size and/or electrostatic forces [26-29]. In an isolated dog lung, Conhaim et al. proposed that the lung epithelial barrier is best described by a three-pore-size model, including a very small number of large pores (400-nm radius), an intermediate number of medium-size pores (40-nm radius), and a very large number of small pores (1.3-nm radius) [28]. If such a theory could be extrapolated to the human lung, it is possible that high molecular size KL-6/MUC1 passes through the alveolar-blood interface, considering that the molecular diameter of KL-6/MUC1 is approximately 200–500 nm [4]. A number of studies have indicated that the surfaces of endothelium, epithelium and basement membranes are covered by negatively charged proteoglycans. Under such conditions, electrostatic forces would affect the movement of charged versus uncharged macromolecules [29,30]. As most lung proteins, particularly mucins, are negatively charged at physiologic pH [31,32], it is very plausible that similar electrostatic repulsion influences their transfer from lung into blood. Hence, we speculate that electrostatic forces would limit the transfer of high molecular size KL-6/MUC1 more efficiently at the alveolar-blood interface, as compared to small molecular size, under healthy conditions. However, if such barrier function is damaged, the high molecular size proteins would more easily pass through the alveolar

blood interface. In our study, the numbers of lymphocytes in BALF tended to be elevated and the serum IL-2 receptor levels were significantly increased in the subjects in which the influx of the high molecular size KL-6/MUC1 was observed. The percentage of lymphocytes in BALF is thought to be a marker of alveolitis [33,34]. Soluble IL-2 receptor was reported to be associated with T-lymphocyte alveolitis [35] . Our results suggest that high molecular size KL-6/MUC1 might transfer from the alveoli to blood circulation as a result of alveolitis.

Cigarette smoke exposure is known to increase the permeability of the lung epithelial/endothelial barrier [36,37]. The mechanisms by which cigarette smoke disrupts epithelial integrity have not been fully defined, but are likely to involve alterations in the function of the tight junctions [38], which normally maintain the polarity of the epithelial cells and limit flow of ions and proteins from one side of the monolayer to the other. In this study, we did not demonstrate statistically significant relationship between smoking status and the leakage behavior of high molecular size KL-6/MUC1. The multivariate linear regression analysis showed that the molecular size of KL-6/MUC1 in serum was only significantly independent determinant of serum KL-6 levels. However, there have been a few reports describing that serum KL-6 levels may be affected by smoking status in healthy controls [17,20]. Further studies to analyze relationships between cigarette smoking and leakage behavior of KL-6/MUC1 and KL-6 levels in healthy controls would be necessary.

The findings of this study are important for both the interpretation of serum KL-6 levels and the consideration of serum marker proteins, such as surfactant protein (SP)-D [39], that originate in lung epithelium and have been identified in serum from patients with sarcoidosis and other ILDs. Leth-Larsen et al. reported that SP-D gene polymorphism influences the potential for oligomerization, which results in significantly different SP-D serum levels [40]. Their data strongly indicates that the passage of SP-D protein through the alveolar-blood interface is also dependent on the size of the SP-D protein corresponding to the gene polymorphism. With regard to serum markers in lung diseases, the different behaviors among markers have long been a subject of debate. The molecular size classes that may be specific to each marker protein could explain the different behaviors of these proteins in serum.

Finally, it is noteworthy that the minor allele (i.e., G allele) frequency of rs4072037 may differ with ethnicity. In the Caucasian population, Janssen et al. reported that it was 0.45 [17], which is much higher than our observed frequency of 0.21, and the frequency in the Japanese population reported by HapMap (www.hapmap.org). Thus, the interpretation of serum KL-6 levels may be more complex in the Caucasian population than in Japanese population. This may explain the fact that measurements of KL-6 are not well accepted as diagnostic markers of sarcoidosis and other interstitial lung diseases in most Western countries, in contrast to Japan, where such measurements are routinely used in various clinical settings.

Conclusions

This study has shown that the molecular structural variants of KL-6/MUC1 and its leakage behavior affect changes in serum levels of KL-6 in sarcoidosis. This information will assist in the interpretation of serum KL-6 levels in sarcoidosis. Further studies to examine the factors contributing to the variable increases in serum levels of KL-6 using molecular analysis in other ILDs would be warranted.

Abbreviations

BAL: Bronchoalveolar lavage; BALF: Bronchoalveolar lavage fluid; D_{LCO}: Diffusing capacity of lung for carbon monoxide; ILDs: Interstitial lung diseases; KL-6: Krebs von den Lungen-6; MUC1: Mucin-1; SNP: Single nucleotide polymorphism; SP-D: Surfactant protein-D; VA: Alveolar volume; VC: Vital capacity; VNTR: Variable number of tandem repeat.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MS, YN, SK, MN contributed to the study design, data analysis, data collection, data interpretation, figures and writing of the manuscript. CS and KM contributed to the study design, data interpretation, and writing of the manuscript. EY contributed to sample collection and interpretation of the data and writing of the manuscript. All authors have read and approved the final manuscript.

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Krebs von den Lungen-6 (KL-6) is a prognostic biomarker in patients with surgically resected nonsmall cell lung cancer

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By immunizing mice with a lung adenocarcinoma cell line, we previously established a murine IgG1 monoclonal antibody that recognizes a sialylated sugar chain designated Krebs von den Lungen-6 (KL-6). KL-6 is a high-molecular-weight glycoprotein classified as a human MUC1 mucin. The aim of this study was to determine whether KL-6 expression in tumors correlates with circulating KL-6 levels and whether circulating KL-6 has any prognostic value in patients with surgically resected non-small cell lung cancer (NSCLC). Immunohistochemical analysis of KL-6 expression was performed on 103 NSCLC tissues, and its associations with serum KL-6 levels and survival were examined. We also evaluated whether KL-6 expression patterns and/or serum KL-6 levels could predict prognosis in these NSCLC patients. Immunohistochemical analysis of KL-6 in NSCLC tissues showed that a depolarized KL-6 expression pattern was associated with a high level of circulating KL-6 and a poor prognosis in NSCLC patients who underwent curative surgery. Furthermore, a high circulating KL-6 level was associated with both poorer progression-free survival (PFS) and overall survival (OS), and multivariate analyses confirmed its independent prognostic value for both PFS and OS ($p = 0.041$ and 0.023, respectively). Our data suggest that preoperative serum KL-6 level reflects KL-6 expression patterns in NSCLC tissue, and can serve as a useful prognostic biomarker in NSCLC patients who undergo curative surgery.

Lung cancer is one of the most common malignant tumors in the world, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of reported cases.[1] The Tumor-Node-Metastasis (TNM) staging system for NSCLC is widely used for selecting candidates for surgical intervention and adjuvant chemotherapy, and is also useful for predicting prognosis. Surgical resection is the first choice for treating NSCLC patients when the disease is categorized as stage IA to IIIA. However, even after complete resection, ~30 and 75% of patients with pathological stage IA and stage IIIA, respectively, die within 5 years.[2,3] The presence of occult metastases in these patients at the time of surgery has been proposed, which is supported by a small but significant improvement in the survival rates of NSCLC patients who received chemotherapy following surgical tumor resection.[4–6] An increasing number of studies have reported that evaluation of the malignant potential of cancer cells in addition to TNM staging is useful for more precisely estimating prognosis in NSCLC patients who have undergone surgery. If this evaluation can be conducted using molecules or substances present in the circulation, tremendous clinical benefit can potentially be provided.

By immunizing a mouse with a lung adenocarcinoma cell line, we previously established a murine IgG1 monoclonal antibody (mAb) that recognizes a sialylated sugar chain designated Krebs von den Lungen-6 (KL-6).[7] KL-6 is classified as a MUC1 mucin, and is known to be expressed in regenerating type II pneumocytes.[8–10] As elevated serum KL-6 levels suggest the presence of interstitial pneumonia,[11–15] KL-6 is used in Japan as a serum biomarker for this disease. However, recent studies have implied that KL-6 can also serve as a tumor marker.[16,17] The involvement of KL-6 in tumor progression was first proposed in a study that showed that the presence of serum anti-KL-6 antibody in NSCLC patients is

Key words: KL-6, MUC1, cell surface antigen, biomarker, lung cancer
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correlated with prognosis.[18] This observation is supported by the results of our previous study, which demonstrated an antitumor effect of anti-KL-6 mAb on cancer cell lines through the facilitation of E-cadherin-mediated cell-cell interaction, induced by the capping of MUC1 by the anti-KL-6 mAb.[19,20] Furthermore, an immunohistochemical analysis showed that expression of KL-6 is increased in breast, lung, pancreatic, ovarian, colon, and hepatocellular carcinoma tissues,[7,21–28] and that the KL-6 expression levels in colon and pancreatic carcinomas are inversely correlated with patient survival.[25–28] These observations also support the involvement of KL-6 in tumor progression, and highlight its potential value as a tumor biomarker. Unlike MUC1, however, no detailed analyses of KL-6, such as determination of subcellular localization in tumor cells, have been conducted. In normal epithelium, MUC1 is present predominantly on the apical surface of epithelial cells.[29] Recent observations demonstrated that in various carcinomas, MUC1 is expressed on the circumferential and basal membranes, in the cytoplasm, and on the apical surface of carcinoma cells.[30] Furthermore, aberrant localization of MUC1 is associated with a poorer prognosis in NSCLC patients.[31] These observations suggest that subcellular localization of MUC1 in carcinoma cells is also associated with tumor progression.

Based on these observations, we hypothesized that subcellular localization of KL-6 might be associated with malignant potential of NSCLC and/or elevated circulating KL-6 levels. To test this hypothesis, we analyzed the relationships between the subcellular localization of KL-6 in tumors, KL-6 serum levels, and the various clinical features of patients with surgically resected NSCLC. Furthermore, compared to other forms of MUC1, KL-6 has a high probability of being detected in the circulation; therefore, to assess the clinical significance of circulating KL-6 levels in NSCLC patients, the correlation between serum KL-6 levels and prognosis were evaluated in the same study group.

Material and Methods

Patients

The study group consisted of 103 patients with NSCLC (38 females and 65 males) who underwent curative surgery at Hiroshima University Hospital (Hiroshima, Japan) between 2000 and 2008. The inclusion criteria were almost identical to those described in our previous studies.[32–36] In brief, inclusion criteria were: (*i*) age ≥20 years, (*ii*) no significant abnormalities in liver or kidney function, and (*iii*) absence of active interstitial lung disease. The final diagnosis of NSCLC was made histologically on surgically excised tissues using WHO criteria.[37] The postsurgical pathologic TNM stage was determined according to the guidelines of the American Joint Committee on Cancer.[38] All of the patients underwent curative surgery without preoperative chemotheory or radiotherapy. For postoperative adjuvant chemotherapy, orally administered UFT (tegafur and uracil) was selected for patients with pathologic stage IB adenocarcinoma, and platinum-based chemotherapy was selected for patients with histologically confirmed lymph node metastasis. The ages of the patients ranged from 43 to 91 years (median age, 67 ± 10.3 years). The histological types of the 103 NSCLC patients were as follows: 78 adenocarcinomas (ADC), 16 squamous cell carcinomas (SCC), 1 large cell carcinoma (LCC), and 8 adenosquamous cell carcinomas (ASC). Local recurrence was defined as recurrence within the bronchial stump, staple line, ipsilateral hilar, or mediastinal lymph nodes as previously described.[39] Distant recurrence was defined as the appearance of tumors in the ipsilateral lung, contralateral lung, or other organs such as adrenal glands, brain, and bone. In addition, 68 patients with interstitial lung diseases (ILDs) and 102 healthy volunteers (61 smokers and 41 nonsmokers) were enrolled as controls in this study. Serum samples were obtained with informed consent from all 103 NSCLC patients just before surgery, the patients with ILDs, and the healthy volunteers at enrollment and were stored at −80°C. This study and the use of all clinical materials mentioned were approved by the individual institutional Ethical Committees.

Purification of anti-KL-6 monoclonal antibody

The anti-KL-6 mouse IgG_1 monoclonal antibody (mAb) was purified from the ascites collected from mice bearing anti-KL-6 mAb-producing hybridomas as previously described,[7] using a protein A affinity column (Affi Gel Protein A MAPS II Kit; Bio-Rad, Hercules, CA).

Immunohistochemistry

Immunohistochemical analysis of KL-6 expression was performed on tissue sections prepared from paraffin blocks of surgically resected lung tissue as described previously.[32–36] The slides were immersed in Target Retrieval Solution, Citrate pH 6 (Dako Japan, Tokyo, Japan) and boiled at 108°C for 15 min in an autoclave for antigen retrieval. After the blocking of endogenous peroxidase activity with 0.03% H_2O_2 for 30 min, a mouse anti-human KL-6 mAb was added to the sections. Sections were incubated with a secondary antibody, HRP-labeled anti-mouse IgG, followed by the addition of a substrate-chromogen, and was then counterstained with hematoxylin. Three independent investigators assessed the staining patterns of KL-6 without prior knowledge of the clinicopathological data. The intensity and pattern of staining were evaluated in 10 microscopic fields that were randomly chosen from the area of tumor, or in the entire area if the tumor tissue was comprised of less than 10 fields. The staining intensity of KL-6 was first classified as negative or positive. If positive KL-6 staining was observed in the tumor tissue, the staining pattern was subsequently classified as apical membrane, circumferential membrane, or cytoplasm, as described previously.[28,30,31]

Measurement of serum KL-6 levels

Serum KL-6 levels were measured by a sandwich-type electrochemiluminescence immunoassay (ECLIA) using a

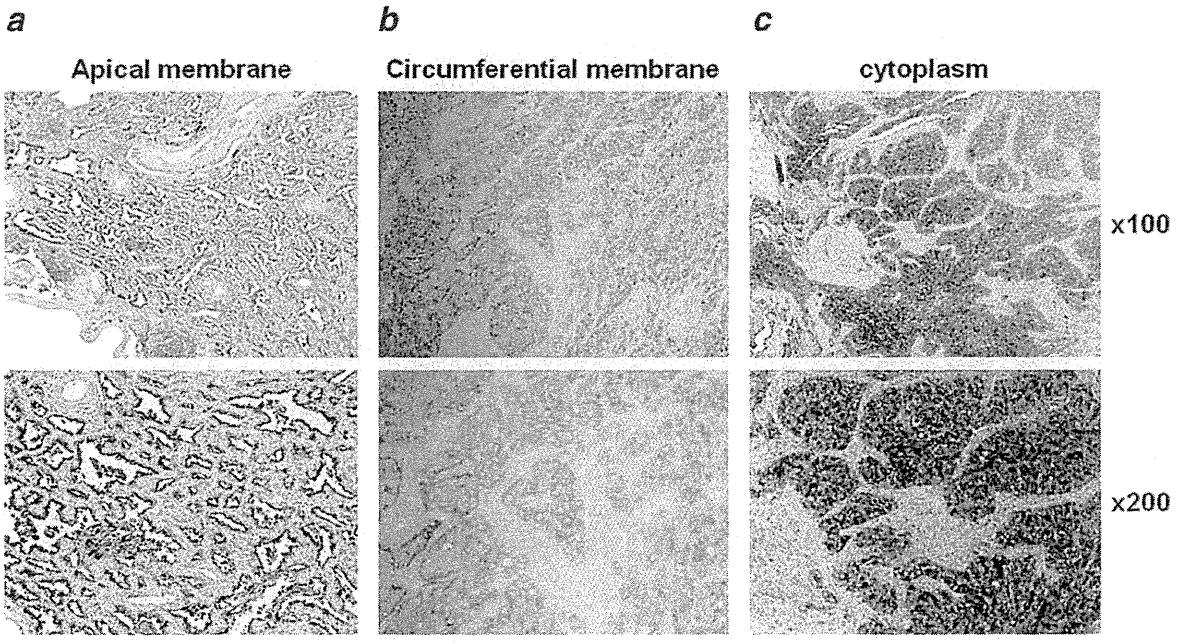

Figure 1. Representative examples of KL-6 staining patterns in NSCLC (top panels, X100; bottom panels, X200): apical membrane (*a*), circumferential membrane (*b*), and cytoplasm (*c*) patterns are shown.

Picolumi 8220 Analyzer (Sanko Junyaku, Tokyo, Japan), as previously described.[10] The serum sample was incubated with anti-KL-6 antibody-coated magnetic beads prior to separation by a magnetic rack. The ruthenium-labeled anti-KL-6 antibody was then added to the beads as a second antibody following a wash with PBS. The reaction mixture was placed into the electrode, and the photons emitted from the ruthenium were measured with a photomultiplier.

Measurement of the serum carcinoembryonic antigen (CEA) and cytokeratin 19 fragments (CYFRA 21-1) levels

The serum levels of CEA and CYFRA 21-1 were measured using commercially available electrochemiluminescence immunoassay systems for CEA (Abbott Diagnostics, Tokyo, Japan) and for CYFRA21-1 (Roche Diagnostics Corp Indianapolis, IN).

Statistical analysis

All statistical analyses were performed using a statistical software package (SPSS for Windows, version 12.0; SPSS Inc; Chicago, IL). Differences between groups were analyzed using the Mann-Whitney U-test or Fisher's exact test. To test differences among the variables evaluated prior to and after surgical resection, the Wilcoxon test was used. Progression-free survival (PFS) was defined as the interval starting from the date of surgery to the date of documented disease progression, death from any cause, or last follow-up. Overall survival (OS) was defined as the interval starting from the date of surgery to the date of death from any cause, or last follow-up. Serum KL-6 levels were further analyzed for their ability to predict prognosis by receiver operating characteristic (ROC) curves and Kaplan-Meier curves. The upper left corner coordinate point of the ROC curve was determined as an optimal cut-off value to discriminate survivors from non-survivors. Survival curves and 95% confidence intervals (CIs) were analyzed by the Kaplan-Meier method, and differences between groups were compared with the log-rank test. The risk factors associated with patient prognosis were evaluated using the Cox proportional hazards regression model with a step-down procedure. Only variables that were statistically significant in the univariate analysis were evaluated by multivariate analysis. The criterion for removing a variable was the likelihood ratio statistic, which was based on the maximum partial likelihood estimate (default *p*-value of 0.05 for removal from the model).

Results

Immunohistochemical staining pattern of KL-6 in tumor tissues and association between clinical outcomes and prognosis in NSCLC patients

To determine whether KL-6 is expressed in NSCLC tissues and whether KL-6 expression is associated with clinical characteristics in NSCLC patients, an immunohistochemical analysis of KL-6 expression was performed on 103 surgically resected NSCLC tissues. Interestingly, KL-6 expression was observed at the apical membrane (Fig. 1*a*), circumferential

Table 1. Subcellular KL-6 expression pattern in NSCLC tissues

	KL-6 staining pattern			
Category	Apical membrane	Circumferential membrane	Cytoplasm	Japanese $n = 103$
Polarized	Positive	Negative	Negative	41
Depolarized	Negative	Positive	Negative	3
	Negative	Negative	Positive	16
	Negative	Positive	Positive	7
	Positive	Negative	Positive	33
	Positive	Positive	Positive	3

Table 2. Association between KL-6 expression pattern and clinical characteristics in the 103 patients with surgically resected NSCLC

			KL-6 expression pattern		
Variables	*n*	(%)	Polarized expression $n = 41$	Depolarized expression $n = 62$	*p*-value
Age, years					
<65	43	41.7	18	25	0.839
≥65	60	58.3	23	37	
Gender					
Male	65	63.1	20	45	0.021[+]
Female	38	36.9	21	17	
Histologic type					
ADC	78	75.7	39	39	<0.001[1,+]
SCC	16	15.5	2	14	
Others	9	8.8	0	9	
T factor					
1	56	54.4	29	27	0.009[+]
2+3	47	45.6	12	35	
N factor					
0	86	83.5	40	46	0.002[+]
1+2	17	16.5	1	16	
Smoking history					
Never	38	36.9	20	18	0.060[2]
Former	28	27.2	13	15	
Current	37	35.9	8	29	
Recurrence pattern					
Distant recurrence	23	22.3	4	19	0.015[3,+]
Local recurrence	10	9.7	1	9	
No recurrence	70	68.0	36	34	

[1]among ADC, SCC, and Others. [2]Never versus others. [3]Distant recurrence versus others. [+] $p < 0.05$ (Fisher's exact test or Chi-square test)
Abbreviations: ADC, adenocarcinoma; SCC: squamous cell carcinoma, Others: adenosquamous cell carcinoma and large cell carcinoma.

membrane (Fig. 1*b*), and/or cytoplasm (Fig. 1*c*) in all 103 NSCLC tissues. Regarding the staining intensity, we could not visually detect apparent differences between the sites of KL-6 expression or the types of histology (data not shown). As shown in Table 1, KL-6 expression patterns were divided into two categories: polarized expression was defined as apical staining only, while depolarized expression was defined as either circumferential membrane or cytoplasm staining. The category of KL-6 expression pattern defined by three independent investigators matched in 97 of 103 (94.1%) studied cases. The unmatched or equivocal cases were re-examined and a consensus category was decided by the three

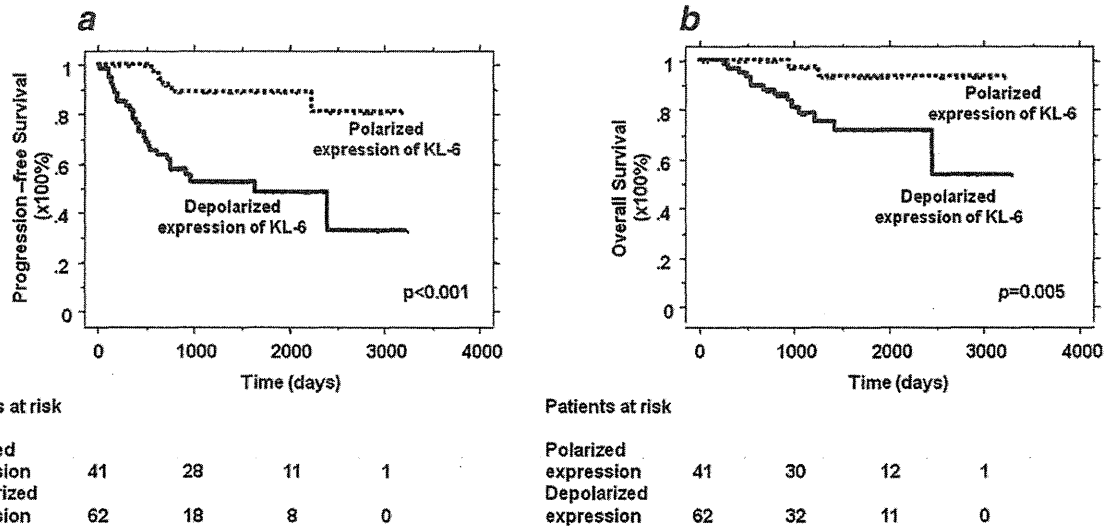

Figure 2. Progression-free survival (*a*) and overall survival (*b*) in all 103 patients with surgically resected NSCLC in relation to KL-6 expression pattern in NSCLC tissues.

investigators. In total, 41 (39.8%) and 62 (60.2%) NSCLC tissues were categorized as having polarized expression and depolarized expression, respectively. The depolarized KL-6 expression pattern was significantly associated with gender (higher in males; $p = 0.021$ by Fisher's exact test), histological type (difference among ADC, SCC, and others; $p < 0.001$ by Chi-square test), *T* factor (higher in T_{2+3}; $p = 0.009$ by Fisher's exact test), *N* factor (higher in N_{1-2}; $p = 0.002$ by Fisher's exact test) and recurrence pattern (higher in distant metastasis; $p = 0.015$ by Fisher's exact test; Table 2). As shown in Figure 2*a* and *b*, PFS and OS of NSCLC patients with a depolarized KL-6 expression pattern were significantly poorer than those of patients with polarized KL-6 expression ($p < 0.001$ and $p = 0.005$, respectively, by log-rank test).

Association between serum KL-6 level and tumor tissue KL-6 staining pattern

To determine whether serum KL-6 levels were elevated in NSCLC patients, serum KL-6 levels were compared among the NSCLC patients, the ILD patients, the nonsmoker controls, and the smoker controls. The serum KL-6 levels (mean ± SD) were significantly higher in the NSCLC patients (325 ± 189 U/mL) than those in the nonsmoker (236 ± 86 U/mL) and smoker controls (232 ± 79 U/mL; $p = 0.043$ and $p = 0.011$, respectively), whereas the serum levels of KL-6 were significantly higher in the ILD patients (1600 ± 1067 U/mL) than those in the NSCLC patients, the nonsmoker controls, and the smoker controls ($p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). However, there was no significant difference between the nonsmokers and smokers.

The optimal cut-off serum levels for KL-6, CEA, and CYFRA21-1 to discriminate survivors from non-survivors were determined as 400 U/mL, 3.5 ng/mL, and 2.0 ng/mL, respectively, using the upper left corner coordinate point of each ROC curve drawn for KL-6, CEA, and CYFRA21-1. Based on this value, patients were divided into a high KL-6 group (serum KL-6 level ≥400 U/mL) and a normal KL-6 group (serum KL-6 level <400 U/mL). High serum KL-6 levels were observed in 23 (22.3%) of the total 103 patients and in 19 (22.1%) of the 86 patients with node-negative NSCLC. Among clinicopathological variables, histological type (difference among ADC, SCC, and others; $p = 0.036$ by Chi-square test) and recurrence pattern (higher in distant metastasis; $p = 0.010$ by Fisher's exact test) were significantly associated with serum KL-6 level (Table 3).

To confirm that circulating KL-6 was derived from tumors, the preoperative and postoperative (2 months after surgery) serum KL-6 levels were compared in 25 patients whose serum samples were available at both time points. As shown in Figure 3*a*, circulating KL-6 levels significantly decreased after surgical tumor resection ($p < 0.001$ by Wilcoxon test). In addition, a statistical analysis revealed that the depolarized KL-6 expression pattern in tumors was associated with a higher level of circulating KL-6 ($p = 0.003$ by the Mann-Whitney *U* test; Fig. 3*b*).

Association between serum KL-6 level and prognosis in NSCLC patients

The PFS and OS of NSCLC patients with high serum KL-6 levels were also significantly poorer than those of patients with normal serum KL-6 levels ($p = 0.003$ and $p < 0.001$, respectively, by log-rank test) (Fig. 3*c* and *d*). Therefore, to determine the prognostic importance of clinical characteristics and serum KL-6 levels in patients with surgically resected

Table 3. Association between serum levels of KL-6 expression and clinical characteristics in the 103 patients with surgically resected NSCLC

Variables	*n*	(%)	Serum levels of KL-6		*p*-value
			Low KL-6 *n* = 80	High KL-6 *n* = 23	
Age, years					
<65	43	41.7	37	6	0.098
≥65	60	58.3	43	17	
Gender					
Male	65	63.1	47	18	0.140
Female	38	36.9	33	5	
Histologic type					
ADC	78	75.7	64	14	0.036[1,+]
SCC	16	15.5	12	4	
Others	9	8.8	4	5	
T factor					
1	56	54.4	46	10	0.247
2+3	47	45.6	34	13	
N factor					
0	86	83.5	67	19	1.000
1+2	17	16.5	13	4	
Smoking history					
Never	38	36.9	33	5	0.140[2]
Former	28	27.2	20	8	
Current	37	35.9	27	10	
Recurrence pattern					
Distant recurrence	23	22.3	13	10	0.010[3,+]
Local recurrence	10	9.7	8	2	
No recurrence	70	68.0	59	11	

[1]Among ADC, SCC, and Others. [2]Never versus others. [3]Distant recurrence versus others. [+] $p < 0.05$ (Fisher's exact test or Chi-square test).
Abbreviations: ADC, adenocarcinoma; SCC: squamous cell carcinoma, Others: adenosquamous cell carcinoma and large cell carcinoma

NSCLC, we performed a Cox proportional hazards regression analysis on the parameters listed in Tables 4 and 5. Univariate analyses revealed that pT stage (odds ratio, 2.338; 95% CI, 1.147−4.765; $p = 0.019$), pN stage (odds ratio, 2.575; 95% CI, 1.193−5.556; $p = 0.016$), serum KL-6 level (odds ratio, 2.818; 95% CI, 1.383−5.740; $p = 0.004$), serum CEA level (odds ratio, 2.506; 95% CI, 1.129−5.561; $p = 0.024$), and serum CYFRA21-1 level (odds ratio, 2.165; 95% CI, 1.068−4.387; $p = 0.032$) were significant prognostic factors for PFS (Table 4); and pN stage (odds ratio, 3.768; 95% CI, 1.360−10.440; $p = 0.011$), serum KL-6 level (odds ratio, 4.789; 95% CI, 1.790−12.814; $p = 0.002$) and serum CYFRA21-1 levels (odds ratio, 3.778; 95% CI, 1.2011−11.885; $p = 0.023$) were significant prognostic factors for OS (Table 5). Multivariate analyses demonstrated serum KL-6 level (odds ratio, 2.192; 95% CI, 1.031−4.662; $p = 0.041$) and pN stage (odds ratio, 2.264; 95%CI, 1.007−5.091; $p = 0.048$) to be an independent prognostic factor for PFS (Table 4); and, serum KL-6 level (odds ratio, 3.378; 95% CI, 0.774−9.652; $p = 0.023$) and pN stage (odds ratio, 2.987; 95% CI, 1.024−8.712; $p = 0.045$) as independent prognostic factors for OS (Table 5).

Discussion

This study supports the hypothesis that KL-6 can serve as a prognostic biomarker for NSCLC. Immunohistochemical analysis revealed that KL-6 was expressed in all of the NSCLC tissues used in the present study. In addition, a depolarized KL-6 expression pattern in NSCLC tissue was shown to be associated with high circulating KL-6 levels and a poor prognosis in NSCLC patients who underwent curative surgery. Furthermore, a Cox proportional hazards regression analysis demonstrated that serum KL-6 level was an independent prognostic factor for both PFS and OS in patients with surgically resected NSCLC.

First, whether or not the circulating KL-6 observed in NSCLC patients is derived from tumors needs to be addressed. In ILD, the primary cellular source of KL-6 has

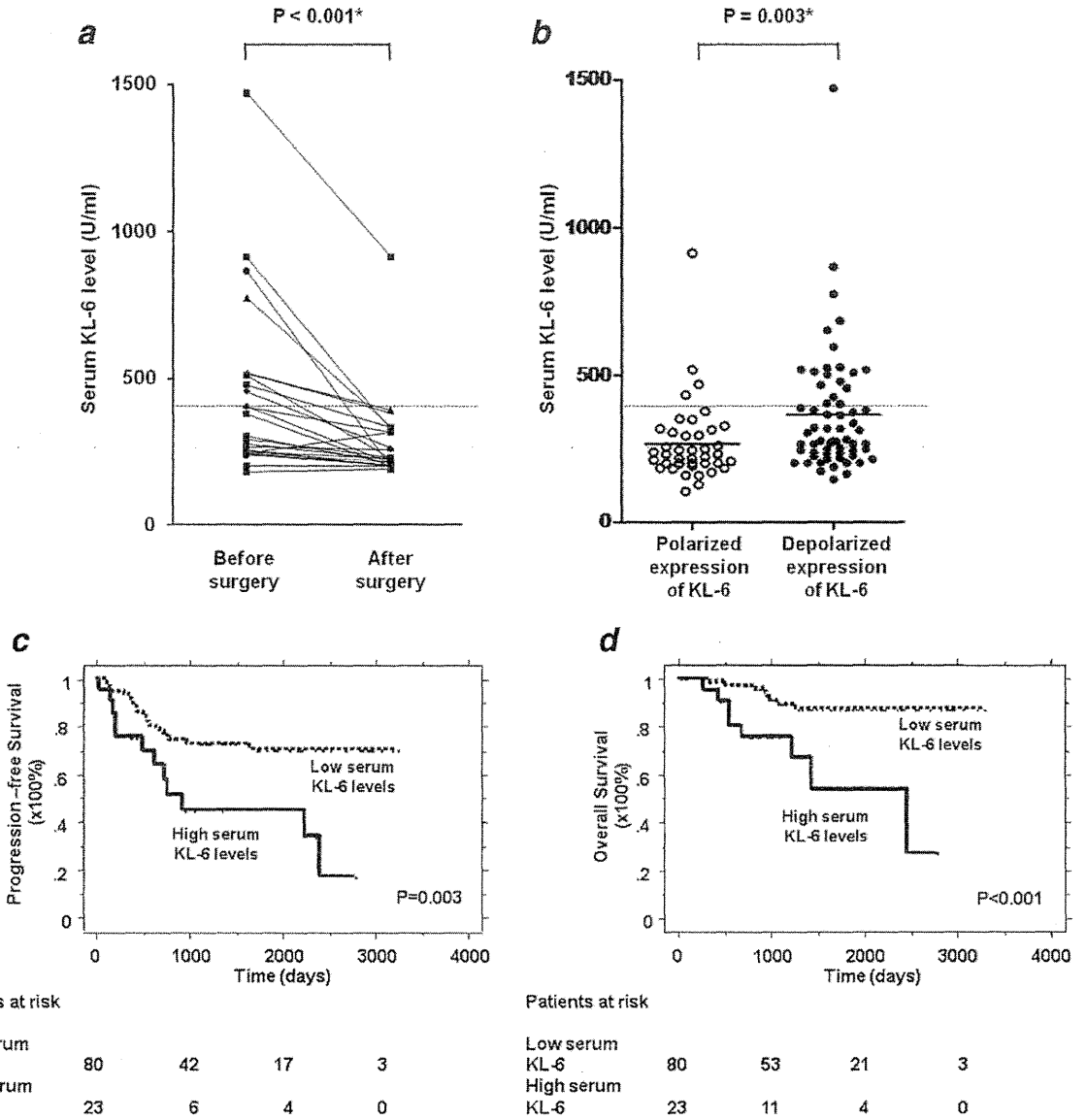

Figure 3. Serum KL-6 levels in patients with surgically resected NSCLC. (*a*) Changes in serum KL-6 levels before and after surgery in the 25 patients whose serum samples were available at both time points. (*b*) The association between preoperative KL-6 serum levels and KL-6 expression pattern in primary tumor tissues in all 103 patients with surgically resected NSCLC. Progression-free survival (*c*) and overall survival (*d*) in relation to preoperative KL-6 serum levels in the 103 patients with surgically resected NSCLC.

been shown to be type II pneumocytes.[11] KL-6 is immunohistochemically detectable in the epithelial cells of pancreatic and mammary ducts.[8] Although our previous studies have already shown that KL-6 levels are elevated in sera from NSCLC patients,[7] the cellular origin of circulating KL-6 in NSCLC patients has not been clearly demonstrated. In this study, serum KL-6 levels were found to decrease dramatically after surgical resection of primary tumors. In addition, KL-6 was immunohistochemically detectable in all of the analyzed NSCLC tissues. These results implied that the primary tumor was the origin of circulating KL-6 in NSCLC patients. Furthermore, immunohistochemical analysis of KL-6 revealed two KL-6 staining patterns in NSCLC tissues distinguished by subcellular localization: polarized and depolarized KL-6 expression patterns. In general, MUC1 mucin is present on the apical surface of the normal secretory epithelia. In malignant tissues, however, this apical polarization is frequently lost, resulting in the localization of MUC1 throughout the

Table 4. Cox proportional hazards model analysis of progression-free survival in patients with surgically resected NSCLC

Variables	Hazards ratio	95% CI	Unfavorable/Favorable	*P*-value
Univariate analysis				
Age (years)	1.104	0.549–2.221	≥65/<65	0.781
Gender	1.041	0.729–1.486	Male/Female	0.825
Histology	1.733	0.840–3.577	Others/ADC	0.137
pT stage	2.338	1.147–4.765	pT_{2+3}/pT_1	0.019*
pN stage	2.575	1.193–5.556	pN_{1+2}/pN_0	0.016*
Smoking history	1.080	0.530–2.198	Smoker/Never	0.833
Serum KL-6 level	2.818	1.383–5.740	≥400/<400	0.004*
Serum CEA level	2.506	1.129–5.561	≥3.5/<3.5	0.024*
Serum CYFRA21-1 level	2.165	1.068–4.387	≥2.0/<2.0	0.032*
Multivariate analysis				
pT stage	1.616	0.749–3.489	pT_{2+3}/pT_1	0.221
pN stage	2.264	1.007–5.091	pN_{1+2}/pN_0	0.048
Serum KL-6 level	2.192	1.031–4.662	≥400/<400	0.041*
Serum CEA level	2.156	0.940–4.965	≥3.5/<3.5	0.070
Serum CYFRA21-1 level	1.575	0.754–3.290	≥2.0/<2.0	0.277

*$p < 0.05$ (Cox proportional hazards regression model with a step-down procedure).
Abbreviations: ADC, adenocarcinoma; Others, squamous cell carcinoma, adenosquamous cell carcinoma, and large cell carcinoma

Table 5. Cox proportional hazards model analysis of overall survival in patients with surgically resected NSCLC

Variables	Hazards ratio	95% CI	Unfavorable/Favorable	*p*-value
Univariate analysis				
Age (years)	1.714	0.595–4.936	≥65/<65	0.318
Gender	1.055	0.635–1.752	Male/Female	0.836
Histology	2.154	0.781–5.944	Others/ADC	0.138
pT stage	1.741	0.645–4.695	pT_{2+3}/pT_1	0.273
pN stage	3.768	1.360–10.440	pN_{1+2}/pN_0	0.011*
Smoking history	1.109	0.402–3.060	Smoker/Never	0.842
Serum KL-6 level	4.789	1.790–12.814	≥400/<400	0.002*
Serum CEA level	1.811	0.628–5.217	≥3.5/<3.5	0.272
Serum CYFRA21-1 level	3.778	1.201–11.885	≥2.0/<2.0	0.023*
Multivariate analysis				
pN stage	2.987	1.024–8.712	pN_{1+2}/pN_0	0.045*
Serum KL-6 level	3.378	0.774–9.652	≥400/<400	0.023*
Serum CYFRA21-1 level	2.587	0.774–8.644	≥2.0/<2.0	0.123

*$p < 0.05$ (Cox proportional hazards regression model with a step-down procedure).
Abbreviations: ADC, adenocarcinoma; Others, squamous cell carcinoma, adenosquamous cell carcinoma, and large cell carcinoma.

cell membrane and in the cytoplasm.[28–31] Because MUC1 mediates anti-adhesive activity by interfering with cell-to-cell and/or cell-to-extracellular matrix interactions, aberrant subcellular expression of MUC1 facilitate detachment of cancer cells from the primary tumor.[11] We believe that this scenario can be applied to KL-6 as well, thereby accounting for the poorer survival observed in patients with NSCLC tumors displaying depolarized KL-6 expression. In addition, we also demonstrated that depolarized expression pattern of KL-6 in the resected tumor was associated with high preoperative serum level of KL-6. As far as we are aware, this is the first study that reports the association between expression pattern of a MUC1-related molecule in tumor and its circulating level.

A novel finding from this study is that preoperative circulating KL-6 level can serve as a prognostic biomarker in NSCLC patients who undergo curative surgery. The optimal cut-off level that could discriminate survivors from non-survivors was established by using ROC curves, and patients with serum KL-6 levels below 400 U/mL were shown to have significantly favorable PFS and OS compared to patients with serum KL-6 levels above 400 U/mL. Furthermore, the Cox proportional hazards regression analysis demonstrated serum KL-6 level to be an independent prognostic factor for both PFS and OS in NSCLC patients who underwent curative surgery. On the other hands, neither CEA nor CYFRA21-1 was a significant independent prognostic factor for PFS and OS, suggesting that KL-6 is a more sensitive serum biomarker than CEA and CYFRA21-1 for predicting clinical outcome of NSCLC patients who undergo curative operation. Regarding the association between serum KL-6 level and prognosis in NSCLC patients, we previously reported that a pretreatment serum level of KL-6 serves as an independent prognostic factor in advanced or refractory NSCLC patients treated with orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs).[17] Although 6 of 103 patients were treated with EGFR-TKIs following postoperative recurrence, none of the patients received EGFR-TKI therapy as postoperative adjuvant chemotherapy. Since survival of NSCLC patients following curative surgery primarily depends on the presence or absence of occult metastases, NSCLC patients with depolarized KL-6 expression in tumors and high levels of circulating KL-6 are therefore more likely to have occult metastases at the time of surgery. In fact, the depolarized KL-6 expression and high serum KL-6 levels were associated with distant recurrence after the surgery in the present study. These results also suggest that KL-6 expression pattern in tumors and serum KL-6 level may be able to be used for selecting patients who should receive adjuvant chemotherapy.

Compared to detecting biomarkers in tumors by immunohistochemical analysis or polymerase chain reaction (PCR), measurement of circulating biomarkers can be more rapid, reproducible, and inexpensive. Several lines of evidence suggest that overexpression of MUC1 in tumors is associated with shorter survival in patients with surgically resected NSCLC.[31,42,43] However, no circulating MUC1-related biomarkers other than KL-6 have been reported. Therefore, KL-6 can be regarded as a novel MUC1-associated serum biomarker for NSCLC. The sensitivity of KL-6 as an indicator of NSCLC was poorer compared to conventional serum NSCLC biomarkers, such as CEA and cytokeratin 19 fragments (CYFRA21-1; data not shown). This suggests that circulating KL-6 is not valuable as a diagnostic biomarker. However, a high preoperative KL-6 serum level was demonstrated to be an independent prognostic factor for OS and PFS in NSCLC patients. These results strongly suggest that KL-6 is a more useful prognostic factor for NSCLC patients who undergo curative surgery.

Although promising results were obtained, we are aware that this study has some limitations. First, the number of patients was not sufficient to perform a valid statistical analysis. Second, the association between KL-6 expression and particular molecular features of NSCLC such *EGFR* mutation was not evaluated. To investigate the significance of KL-6 expression and its association with various clinical variables including *EGFR* mutation status, we believe that a prospective study in a larger number of patients with surgically resected NSCLC is required.

In conclusion, the results of the present study indicate that both tumor KL-6 expression pattern and circulating KL-6 level are useful for predicting survival of NSCLC patients who have undergone curative surgery.

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