Pre-operative evaluation of circulating KL-6 levels as a biomarker for epithelial ovarian carcinoma and its correlation with tumor MUC1 expression

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Abstract. Krebs von den Lungen-6 (KL-6), a mucinous sialylated sugar chain on human mucin-1 glycoprotein (MUC1), is a diagnostic marker for interstitial lung diseases. Furthermore, elevated serum KL-6 levels have been observed in certain malignant tumor types of epithelial origin. The expression of MUC1 has been observed in patients with epithelial ovarian cancer (EOC) and is considered a potential therapeutic target. In the present study, KL-6 serum levels were investigated in patients clinically suspected of having malignant ovarian tumors. A total of 219 patients were enrolled in the study, which analyzed their serum KL-6 levels in addition to tumor expression of MUC1 using immunohistochemistry. High serum KL-6 levels were predominantly observed in patients with EOC, and did not occur in patients with benign or borderline tumors. The level of serum KL-6 was highly correlated with tumor stage, grade and histological type, and demonstrated superior sensitivity for the detection of ovarian cancer compared with that of serum cancer antigen 125. High serum KL-6 was significantly associated with shorter progression-free survival. In addition, tumor MUC1 expression status was significantly correlated with serum KL-6 levels. These data suggest that serum KL-6 may be a useful, non-invasive biomarker surrogate for tumor MUC1 expression in future clinical trials of MUC1-targeted therapy.

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Abbreviations: KL-6, Krebs von den Lungen-6; MUC1, human mucin-1 glycoprotein; ILD, interstitial lung disease; FFPE, formalin-fixed paraffin-embedded; mAb, monoclonal antibody; ROC, receiver operator characteristic; EOC, epithelial ovarian cancer; PFS, progression-free survival; OS, overall survival

Key words: ovarian cancer, Krebs von den Lungen-6, human mucin-1 glycoprotein, serum biomarker, diagnostic marker

Introduction

Ovarian cancer is the fifth leading cause of cancer-associated mortality for women in the USA (1). It is the most fatal type of gynecologic malignancy, and is characterized by few early symptoms, widespread peritoneal dissemination and ascites at advanced stages (2). Existing limitations in the detection, diagnosis and treatment of ovarian cancer have contributed to a poor overall survival (OS) rate (2). It is therefore imperative that such difficulties are overcome. The majority of epithelial ovarian cancer (EOC) cases (61%) are diagnosed in the advanced stages, and the mean 5-year survival rate is 27% (2). However, 60-90% of patients diagnosed when they are at stage I/II of the disease survive, depending on the tumor grade; thus, there is an increased possibility for curing the disease in cases of earlier detection and treatment (3,4). Since physical symptoms are almost absent in the early stages of ovarian cancer, efforts to develop assays for blood or urine based non-invasive biomarkers are currently in progress (5). Serum cancer antigen (CA)125 is an ovarian cell surface glycoprotein that has been demonstrated to be elevated in 80% of patients with advanced EOC (6). However, in the early stages of EOC, CA125 possesses a predictive value of 10% (7). This can be attributed to the relatively low specificity of CA125. Elevated CA125 levels have been associated with various non-malignant conditions, including pregnancy, endometriosis, adenomyosis, uterine fibroids, pelvic inflammatory disease, menstruation and benign ovarian cysts (8). Furthermore, abnormal CA125 levels have been associated with other malignancies, including pancreatic (9), breast (10), lung (11), gastric (12) and colorectal cancer (13), particularly when they are associated with peritoneal spreading. CA125 may not be useful in identifying early stages or providing a differential diagnosis for these diseases, as malignancies with peritoneal spreading typically exhibit a high level of CA125. However, CA125 may be suitable for monitoring the response

of patients to chemotherapy and in the early detection of relapse in patients with ovarian cancer (14).

Human mucin-1 glycoprotein (MUC1) is encoded by the *MUC1* gene, which is located on the long arm (q) of chromosome 1 at position 21, and has a molecular weight between 250 and 500 kDa. The transmembrane glycoprotein MUC1 is a well-known target for cancer therapy (15). MUC1 is aberrantly glycosylated and overexpressed in >90% of epithelial cancer types, including ovary, breast, pancreas and lung epithelial cancer (16). Several studies have investigated the association between the expression of MUC1 and EOC (17), and have demonstrated that MUC1 is overexpressed in >90% of late-stage EOC and metastatic lesions (18). MUC1 expression is typically observed in the glandular or luminal epithelial cells of the mammary gland, esophagus, stomach, duodenum, pancreas, uterus, prostate and lungs, and in hematopoietic cells to a lesser extent (15). MUC1 possesses negatively charged sugar branches that are extended such that a physical barrier is created, conferring an anti-adhesive property to MUC1 that limits accessibility and prevents microbial growth (19,20). Such biological differences between tumor and healthy tissue makes MUC1 a promising therapeutic target for cancer, particularly in the context of targeting MUC1 for tumor-specific killing.

MUC1 is currently being investigated in preclinical and clinical trials as a diagnostic and therapeutic target for ovarian cancer (21,22). Full-length MUC1 is comprised of two subunits, the N-terminal (MUC1-N) and C-terminal (MUC1-C); in normal growth conditions, MUC1 remains as a heterodimer (MUC1-N and MUC-C) on the cell surface (15). Soluble MUC1 is generated from cleavage of the extracellular domain by enzymes such as disintegrin and metalloprotease domain-containing protein 17 and matrix metalloproteases (23,24). Cleaved MUC1-N has been identified in the circulation of patients with cancer, and can be used as a biomarker for cancer diagnosis, staging or monitoring relapse following initial therapy (15,23-25).

Krebs von den Lungen-6 (KL-6) is a high-molecular-weight glycoprotein classified as 'cluster 9' (MUC-associated) according to the Third International Workshop on Lung Tumor and Differentiation Antigens (26). The anti-KL-6 monoclonal antibody (mAb) is considered to recognize the specific MUC1 glycopeptide sequence, but the precise glycan structure of the epitope remains unclear (27). In a study by Seko *et al* (28), it was reported that newly-identified O-linked glycans of MUC1, containing 6'-sulfo-galactose/N-acetylgalactosamine, could be the carbohydrate epitopes of the anti-KL-6 mAb. KL-6 was first suggested as a serum tumor marker for pulmonary, breast and pancreatic cancer; however, the diagnostic accuracy of KL-6 was demonstrated to be lower than carcinoembryonic antigen levels, based on the high number of false positives in patients with pulmonary fibrosis (29). Further investigations have demonstrated that KL-6 is elevated in patients with interstitial pneumonia (30). KL-6 has been approved by Japan's National Health Insurance Program as a diagnostic marker for interstitial lung diseases (ILDs) since 1999; presently, KL-6 levels are examined in >2,000,000 samples annually in Japan (29). The results of a study performed by Nakao *et al* (31) identified an association between increased KL-6 levels and ovarian cancer with predominantly intrathoracic lesions. This suggests that KL-6 is a useful tumor marker for ovarian cancer. Thus, further studies are warranted on the diagnostic and prognostic value of KL-6 as a tumor biomarker for EOC in a prospective clinical setting with detailed patient information.

In the present study, there was focus on patients who were clinically suspected to have malignant ovarian tumors, and the serum KL-6 levels were investigated in these patients prior to surgery. Serum KL-6 was evaluated as a diagnostic marker for ovarian cancer and was compared with CA125. Furthermore, tumor MUC1 expression was immunohistochemically examined to investigate the association between circulating KL-6 and tumor MUC1 status. Thus, the results of the present study address the feasibility of using this non-invasive test in future clinical studies of MUC1-targeted therapies for patients with EOC.

Materials and methods

Patients, sera and tumor specimens. The present study was a retrospective analysis, based on a previous prospective study for ovarian cancer biomarkers that was reviewed and approved by the Institutional Review Board of Saitama Medical University International Medical Center (Saitama, Japan); full details are described in Kurosaki *et al* (32). In brief, 219 women were enrolled in the study prior to surgery at Saitama Medical University International Medical Center between December 2010 and March 2013. The patients were clinically suspected of having borderline-to-malignant ovarian tumor, and were thus investigated to validate candidate soluble molecules for ovarian cancer biomarkers. All patients eventually underwent surgery. Of the 219 patients, 132 (56.9%), 40 (17.3%) and 43 (18.6%) patients were pathologically diagnosed with malignant, borderline and benign ovarian tumors, respectively. Furthermore, 4 patients were diagnosed with ovarian metastasis of a primary colorectal cancer. Of the 132 malignant ovarian tumors, 126 tumors were diagnosed with EOC. Of these, 60 (47.5%) were serous carcinoma, 22 (17.5%) endometrioid carcinoma, 34 (27.0%) clear cell carcinoma, 5 (4.0%) mucinous carcinoma and 5 were classed as 'others'. There were 38 cases of EOC (30.2%) at stage I, 21 (16.6%) at stage II, 54 (42.9%) at stage III and 13 (10.3%) at stage IV.

Tissue specimens were collected at the time of surgery, and formalin-fixed paraffin-embedded (FFPE) specimens were stored at room temperature. Patient sera were collected from all patients prior to surgery and stored immediately at -80˚C until required for use in subsequent experiments. Serum CA125 levels were measured at the Central Clinical Laboratory of Saitama Medical University. Relevant clinical and histopathological information were extracted from clinical charts and pathology reports, respectively. The clinical stage of each tumor was determined according to the guidelines provided by the International Federation of Gynecology and Obstetrics (FIGO) staging system (33). The histological type and grade of each tumor were determined by pathologists experienced in gynecologic oncology (Department of Pathology, Saitama Medical University International Medical Center, Saitama, Japan).

Measurement of serum KL-6. Sera from 219 patients were available for the present study. Serum levels of KL-6 were examined using a Picolumi KL-6 kit (EIDIA Co., Ltd., Ibaraki,

Japan), which is a Japan Pharmaceuticals and Medical Devices Agency-approved electrochemiluminescence immunoassay kit (approval no. 21100AMZ00542000). All assay steps were performed in accordance with the manufacturer's protocol and were performed using anonymously coded samples by technicians who were blind to the patients' diagnosis.

Immunohistochemical staining. The NCL-MUC1 antibody (clone Ma695; Leica Microsystems, Ltd., Milton Keynes, UK), a specific antibody that binds to the carbohydrate epitope of the human MUC1 glycoprotein, was used to investigate tumor MUC1 expression in FFPE specimens obtained from 158 EOC tissues, including borderline malignancies. Immunohistochemical staining was performed on slides with 5 μm sections using a BenchMark XT Automated Slide Stainer and an iVIEW DAB Detection kit, according to the manufacturer's protocol (both from Ventana Medical Systems, Inc., Tucson, AZ, USA). Sections were first deparaffinized and hydrated. Subsequently, antigen retrieval was performed using Cell Conditioning Solution 1 (Ventana Medical Systems, Inc.), followed by incubation for 1 h with the aforementioned mouse monoclonal anti-MUC1 primary antibody (1:100 dilution) at room temperature, and incubation for 0.5 h with the aforementioned iVIEW DAB Detection kit at for 0.5 h at 37˚C. Staining was observed under a light microscope and was considered positive if membranous or membranous with cytoplasmic stain was present in tumor cells. Slides were then scored according to the proportion of staining as follows: Negative, <1%; 1+, <25%; 2+, <75%; and 3+, ≥75%. Furthermore, a score of 2+ or 3+ was classified as high tumor MUC1 expression. The scoring of MUC1 staining was blindly evaluated by two independent observers. Any discrepancies were resolved by a joint review using a double-headed microscope.

Statistical analysis. One-way analysis of variance (ANOVA) with Tukey's post hoc analysis, Student's *t*-tests and Mann-Whitney U tests were used to assess the differences between the patient groups. Receiver operator characteristic (ROC) curve analysis was performed to determine the differences between sensitivity and specificity for serum KL-6 or CA125 between the patient groups. The cut-off values for serum KL-6 or CA125 were determined using the Youden's index as the maximum value of (sensitivity+specificity-1). This index was calculated from all points of an ROC curve and used as a criterion for selecting the optimum cut-off value. Survival was assessed using the Kaplan-Meier estimator method, and any significant differences between groups were determined using the log-rank test. All statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Serum KL-6 in patients with malignant, borderline and benign ovarian tumors. A total of 219 patients were enrolled in the present study prior to surgery. These patients were clinically suspected of having borderline-to-malignant ovarian tumors, and eventually underwent surgical intervention (Fig. 1A). Sera from all patients were analyzed for serum KL-6 levels. As illustrated in Fig. 1B, patients with malignant tumors exhibited high levels of serum KL-6 while patients with benign or borderline tumors exhibited low levels of serum KL-6. Despite the small sample size (n=4), patients with ovarian metastasis from colorectal cancer did not exhibit elevated serum KL-6 levels. The median serum KL-6 levels in those with malignant ovarian tumors was 450.5 U/ml [95% confidence interval (CI), 330-890 U/ml], which was significantly higher compared with that of borderline (202.5 U/ml; 95% CI, 189.0-224.0 U/ml) and benign tumors (205.0 U/ml; 95% CI, 180.0-252.0 U/ml) (P<0.01, Tukey's test). Low levels of serum KL-6 were observed in almost all non-epithelial ovarian tumor serum samples (data not shown).

Association between serum KL-6 and clinicopathological factors in patients with epithelial ovarian, fallopian tube and primary peritoneal cancer. Table I summarizes the serum KL-6 levels in association with the clinicopathological features in 126 patients with epithelial cancer. Of these patients, 108 had primary ovarian cancer, 12 had primary fallopian tube cancer and 6 had primary peritoneal cancer. High serum KL-6 levels were identified to be correlated with FIGO stage, histological type, tumor grade, lymph node metastasis and residual tumor size. Patients with cancer in the advanced stages exhibited significantly higher serum KL-6 levels compared with those exhibited by patients in the early stage (P=0.0018). Serum KL-6 levels were significantly higher in serous tumor serum samples compared with those of the non-serous histotype (P=0.0028, Student's *t*-test). Grade 3 tumors exhibited significantly higher serum KL-6 levels compared with those of grade 1 and 2 tumors (P<0.01, Tukey's test). Furthermore, high serum KL-6 levels were observed in patients with lymph node metastases (P=0.0011). A residual tumor size of ≥1 cm demonstrated higher serum KL-6 levels compared with those with no residual tumor size (P<0.001, Tukey's test). No significant correlation was identified between serum KL-6 levels and other clinical variables.

Serum KL-6 as a diagnostic marker for EOC. The diagnostic significance of serum KL-6 in differentiating patients with EOC from patients with pelvic masses suspected as malignant ovarian tumors was investigated. The ability of serum KL-6 in predicting the presence of EOC was compared with that of CA125. Fig. 2A demonstrates the ROC curves for all patients to predict EOC, and Table II summarizes those results. The ROC analysis indicated that serum KL-6 was marginally superior to CA125, with areas under the curve (AUCs) of 0.81 and 0.78 for serum KL-6 and CA125, respectively. Youden's index, a measure that serves as a summary of the ROC curve (34), also indicated that serum KL-6 performed better than CA125, with Youden's index values of 0.55 and 0.48 for serum KL-6 and CA125, respectively. The appropriate cut-off values determined by the Youden's index for serum KL-6 and CA125 to predict EOC were 293 and 225 U/ml, respectively. Regarding EOC, the diagnostic sensitivity, specificity and positive predictive value for serum KL-6 were 70.1, 85.1 and 87.2%, respectively. However, those for CA125 were 58.6, 89.7 and 88.2%, respectively (Table II). Serum KL-6 thus exhibited an improved performance in discriminating between EOCs and other tumors, with higher sensitivity compared with CA125.

Detecting early stage EOC using a serum marker is often difficult due to its lower sensitivity at early stages of

Table I. Correlation between KL-6 serum levels and clinicopathological features in epithelial ovarian cancer patients.

Clinicopathological factor	N (%)	Mean KL-6 (SD), U/ml	P-value
Age, years			
≤60	67 (53.1)	1,310 (1,684)	0.3456[a]
>60	59 (46.9)	1,712 (2,979)	
Menopause			
Yes	89 (70.7)	1,571 (2,598)	0.5931[a]
No	37 (29.3)	1,321 (1,761)	
FIGO stage			
I, II	59 (46.8)	802 (2,518)	0.0018[a]
III, IV	67 (53.2)	2,111 (2,080)	
Primary site			
Ovary	108 (85.7)	1,438 (2,444)	0.6723[b]
Fallopian tube	12 (9.5)	1,632 (1,313)	
Peritoneum	6 (4.8)	2,309 (2,983)	
Histology			
Serous carcinoma	60 (47.5)	2,155 (1,963)	0.0028[c]
Endometrioid carcinoma	22 (17.5)	1,254 (3,997)	
Clear cell carcinoma	34 (27.0)	792 (1,640)	
Mucinous carcinoma	5 (4.0)	265 (74)	
Others	5 (4.0)	722 (442)	
Grade			
1	20 (15.9)	562 (786)	0.0013[b]
2	70 (55.5)	1,176 (1,653)	
3	36 (28.6)	2,645 (3,523)	
Tumor size, mm			
<100	61 (48.4)	1,685 (1,936)	0.0034[a]
≥100	65 (51.6)	1,322 (2,733)	
Ascites cytology			
Positive	78 (61.9)	1,730 (1,907)	0.1627[a]
Negative	48 (38.1)	1,120 (2,974)	
Lymph node metastasis			
Positive	17 (13.5)	3,227 (4,473)	0.0011[a]
Negative	109 (86.5)	1,228 (1,743)	
Residual tumor size, cm			
0	60 (47.6)	767 (1,504)	0.0011[b]
<1	7 (5.6)	891 (958)	
≥1	59 (46.8)	2,314 (2,919)	

[a]Student's *t*-test; [b]One-way analysis of variance; [c]Student's *t*-test (serous vs. non-serous carcinoma). KL-6, Krebs von den Lungen-6; FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation.

diseases (35). CA125, as a marker, exhibits a high false-positive rate for benign and borderline tumors (8). Therefore, the levels of serum KL-6 were compared among localized epithelial ovarian tumors, including epithelial benign tumors, borderline tumors and stage I/II EOCs. Based on the ROC analysis in Fig. 2B and Table II, the sensitivity of serum KL-6 and CA125 in diagnosing stage I/II EOC from benign and borderline tumors was compared. It was observed that KL-6 possessed an improved sensitivity compared with that of CA125 in detecting patients with stage I/II EOC. Using KL-6, 50.8% (30/59) of patients were diagnosed with early stage EOC, while only 32.2% (19/59) patients were identified to be positive for early stage EOC when CA125 was used. In addition, 35% (14/40) of patients with stage I/II EOC who were CA125-negative were identified as positive for serum KL-6. Thus, KL-6 may demonstrate complementary expression with CA125 in patients with early stage EOC.

Association between serum KL-6 and survival in patients with EOC. The prognostic potential of serum KL-6 in patients

Table II. Summary of receiver operator characteristic curve analysis of KL-6 and CA125.

	EOC (all patients) vs. others[a]		EOC (stage I /II) vs. others[a]	
Factor	KL-6	CA125	KL-6	CA125
AUC	0.81	0.78	0.68	0.62
Youden's index	0.55	0.48	0.35	0.22
Cut-off value, U/ml	293	225	293	224
Sensitivity, %	70.1	58.6	50.8	32.2
Specificity, %	85.1	89.7	83.9	89.7
PPV, %	87.2	88.2	68.2	67.9
NPV, %	66.2	60.0	71.6	66.1
Accuracy, %	76.1	71.2	70.5	66.4

KL-6, Krebs von den Lungen-6; CA, cancer antigen; EOC, epithelial ovarian cancer; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value. [a]Includes benign and borderline tumors, and ovarian metastases.

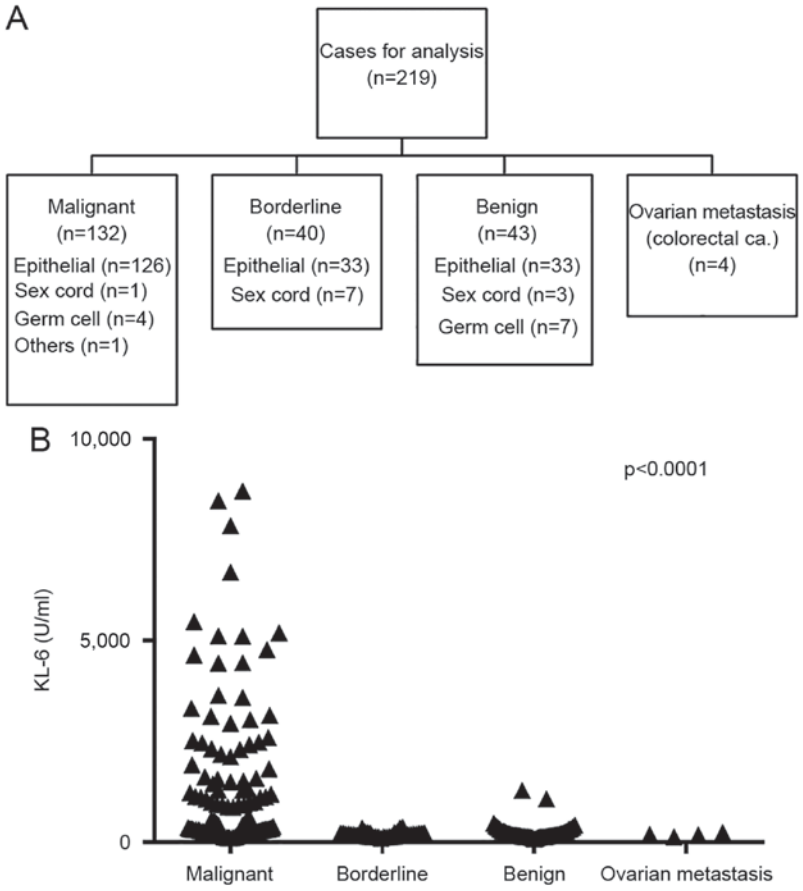

Figure 1. Characteristics of the 219 patients initially included in the study. (A) Diagnoses of the 219 patients who presented with clinical suspicion of a malignant ovarian tumor, as initially included in the present study. (B) Serum KL-6 levels in the types of ovarian tumor, as determined using an electrochemiluminescent immunoassay. One-way analysis of variance was used in the statistical analyses of differences in distribution of KL-6 levels between each group. Ca., cancer; KL-6, Krebs von den Lungen-6.

with EOC was assessed by measuring its levels immediately prior to primary surgery. Of the 126 patients included in the analysis, the median follow-up time for patients who were still alive following the initial diagnosis was 41 months. Compared with patients with higher serum KL-6 levels, patients with lower serum KL-6 levels experienced a significantly longer progression-free survival (PFS) (P<0.0001; Fig. 3A), where serum KL-6 <514.5 U/ml was considered low (median, 514.5 U/ml). However, despite optimizing the cut-off points for OS, including average, median and quartiles, no prognostic association between serum KL-6 levels and OS in the population was determined. Survival analyses were performed on

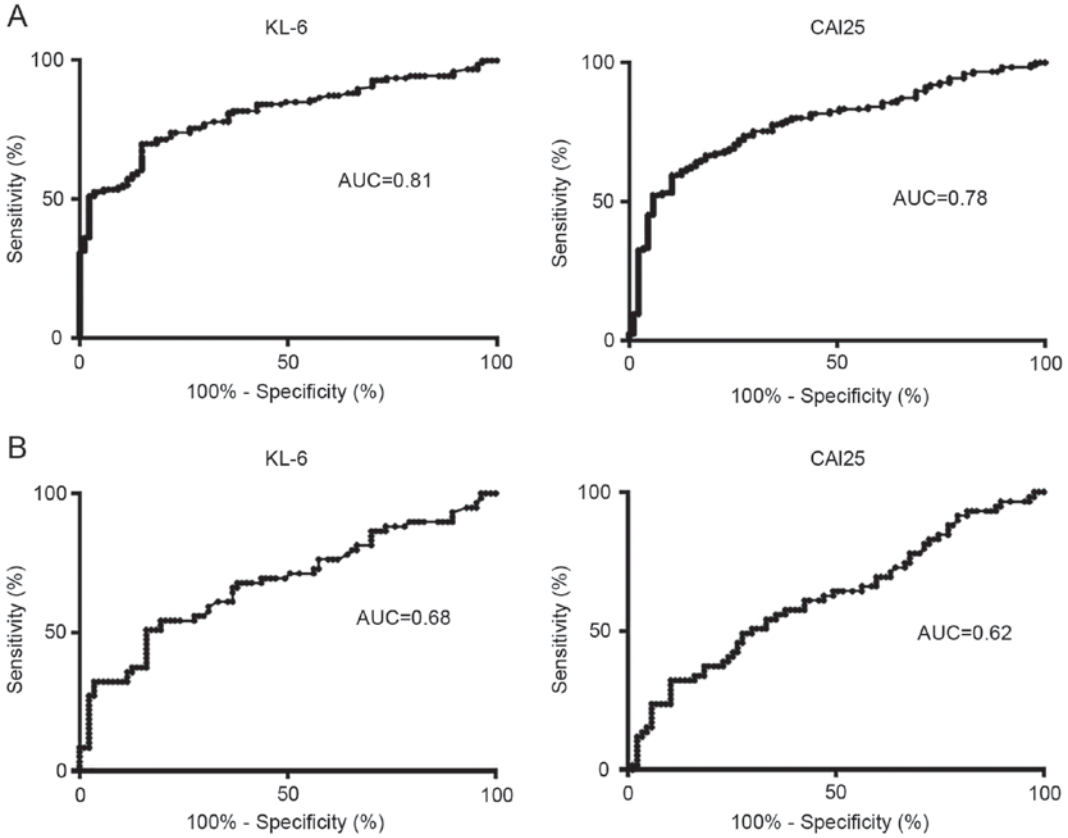

Figure 2. (A) Serum KL-6 and CA125 ROC curves for the prediction of epithelial ovarian, fallopian tube and primary peritoneal cancer. (B) Serum KL-6 and CA125 ROC curves for the prediction of early stage (stages I and II) epithelial ovarian cancer. KL-6, Krebs von den Lungen-6; CA125, cancer antigen 125; AUC, area under the curve; ROC, receiver operator characteristic.

subgroups by stage of disease, as high KL-6 was frequently observed in advanced stage tumors (Table I). As demonstrated in Fig. 3B and C, no correlations between serum KL-6 and clinical outcomes in early and advanced patients with EOC subgroups were identified.

Association between serum KL-6 and tumor MUC1 expression. Several clinical trials are currently investigating the efficacy of MUC1-targeted therapies (21,22). It is important to note, however, that although tumor MUC1 expression is potentially important in patient selection for such targeted therapies, it is often difficult to measure due to limited sample availability, particularly at the time of recurrence (32). In addition, a real-time non-invasive method to monitor the targeted therapy ensures safety and efficacy (36). Consequently, it appeared appropriate to investigate the association between serum KL-6 and tumor MUC1 expression to assess whether serum KL-6 levels could reflect tumor MUC1 status. For this purpose, MUC1 expression in EOC was assessed using immunohistochemistry with a specific antibody that binds to the carbohydrate epitope of the human MUC1 glycoprotein. The representative images demonstrated in Fig. 4A illustrated that tumor MUC1 expression was evident in the majority of EOCs, albeit with variable proportions of staining. Fig. 4B illustrates the association between tumor MUC1 expression and serum KL-6 in the patients with EOC. A correlation was identified, indicating an association between increasing serum KL-6 levels and tumor MUC1 expression status ($P<0.0001$, one-way ANOVA with Tukey's post hoc test). Thus, scores of 2+ and 3+ were defined as high tumor MUC1 expression, and ROC analysis was performed to determine if serum KL-6 could predict high tumor MUC1 expression (Fig. 5).

Based on the cut-off value of 584 U/ml calculated (Table III), 0% (0/9) and 13% (3/23) of patients with negative and 1+ tumor MUC1 expression, respectively, demonstrated serum KL-6 levels above the cut-off value. However, 46.6% (27/58) of patients with 2+ and 80.0% (28/35) of patients with 3+ MUC1 expression exhibited positive serum KL-6 results. A cut-off value of 584 U/ml was able to predict high MUC1 expression with a predictive value of 94.8% in patients with EOC (Table III). Although 26.5% (9/34) and 8.8% (3/34) of patients with borderline malignancy demonstrated 2+ and 3+ tumor MUC1 expression following immunohistochemical analysis, respectively, no serum KL-6 was detected (Fig. 1B). These data suggest that circulating KL-6 may predict the local tumor MUC1 status in a non-invasive manner. Notably, serum KL-6 may potentially serve as a surrogate marker for tumor MUC1 expression in patients with EOC.

Discussion

In the present study, the serum KL-6 levels in patients who were suspected with having malignant ovarian tumors were analyzed. High levels of serum KL-6 were observed in patients with malignant ovarian tumors that did not possess ILDs. However, those with borderline or benign tumors demonstrated

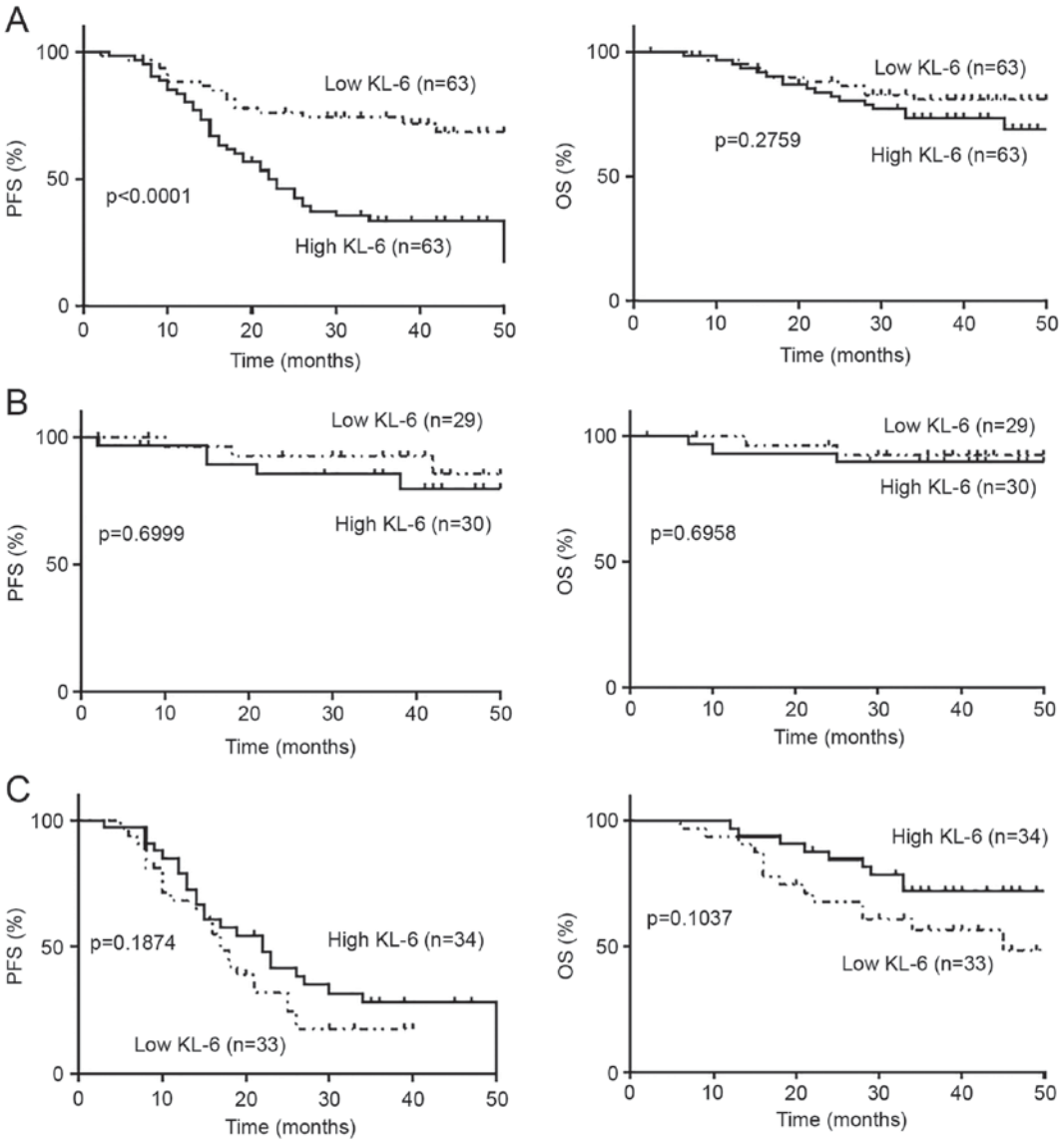

Figure 3. Kaplan-Meier survival curves and corresponding serum KL-6 levels in patients with (A) ovarian cancer in (B) stages I and II and (C) stages III and IV. The log-rank test was used for the statistical analysis of the difference in survival rate between the low and high serum KL-6 groups. The median value (515 U/ml for all patients, 294 U/ml for the early stage subgroup and 1,459 U/ml for the advanced stage subgroup) was the cut-off value between groups for serum KL-6. PFS was significantly different between the low and high serum KL-6 groups in all patients. No survival difference between the high and low serum KL-6 groups was observed in other subgroups. KL-6, Krebs von den Lungen-6; PFS, progression-free survival; OS, overall survival.

either no increase or a marginal increase in serum KL-6 levels. Elevated serum KL-6 levels correlated with FIGO stage, histological type, tumor grade, tumor size, lymph node metastasis and residual tumor size. The results from the ROC analysis indicated that serum KL-6 was more sensitive in predicting EOC than CA125. In addition, it was demonstrated through immunohistochemical staining that serum KL-6 levels correlated with tumor MUC1 expression. This promising result suggests that circulating KL-6 could potentially serve as a biomarker for monitoring disease progression or responses to therapeutics in ovarian cancer and even in patient selection for MUC1-targeted therapies.

Ovarian cancer biomarkers remain an important unmet clinical necessity with regard to early disease detection, predicting prognosis and monitoring therapeutic responses (5). CA125 is currently the most commonly used serum biomarker for monitoring ovarian cancer (37). However, its poor specificity has led to high false-positive rates in screening and early disease detection (35). MUC1 is a glycoprotein that is overexpressed in malignant tumor cells, and has been demonstrated to serve an important role in tumor development, growth, invasion, cellular signal transduction and chemoresistance (38). Serum MUC1 has been investigated as a biomarker for breast cancer and EOC based on antibodies directed against circulating MUC1 antigens (17). There are several assays to detect soluble MUC1 using mAbs such as DF3, HMFG1, HMFG2 and M2C5 (17). Serum MUC1 levels can also be measured with the soluble MUC1 antigens, CA15.3 and CA27.29 (39-41). Fisken *et al* (42) evaluated soluble MUC1 levels using HMFG2 antibody in patients with EOC, and revealed that 45 and 61% of patients with stage I and III EOC, respectively, had elevated serum MUC1. Furthermore, these levels were identified to be significantly correlated with post-operative residual tumor volume (42). A retrospective study by Havrilesky *et al* (37)

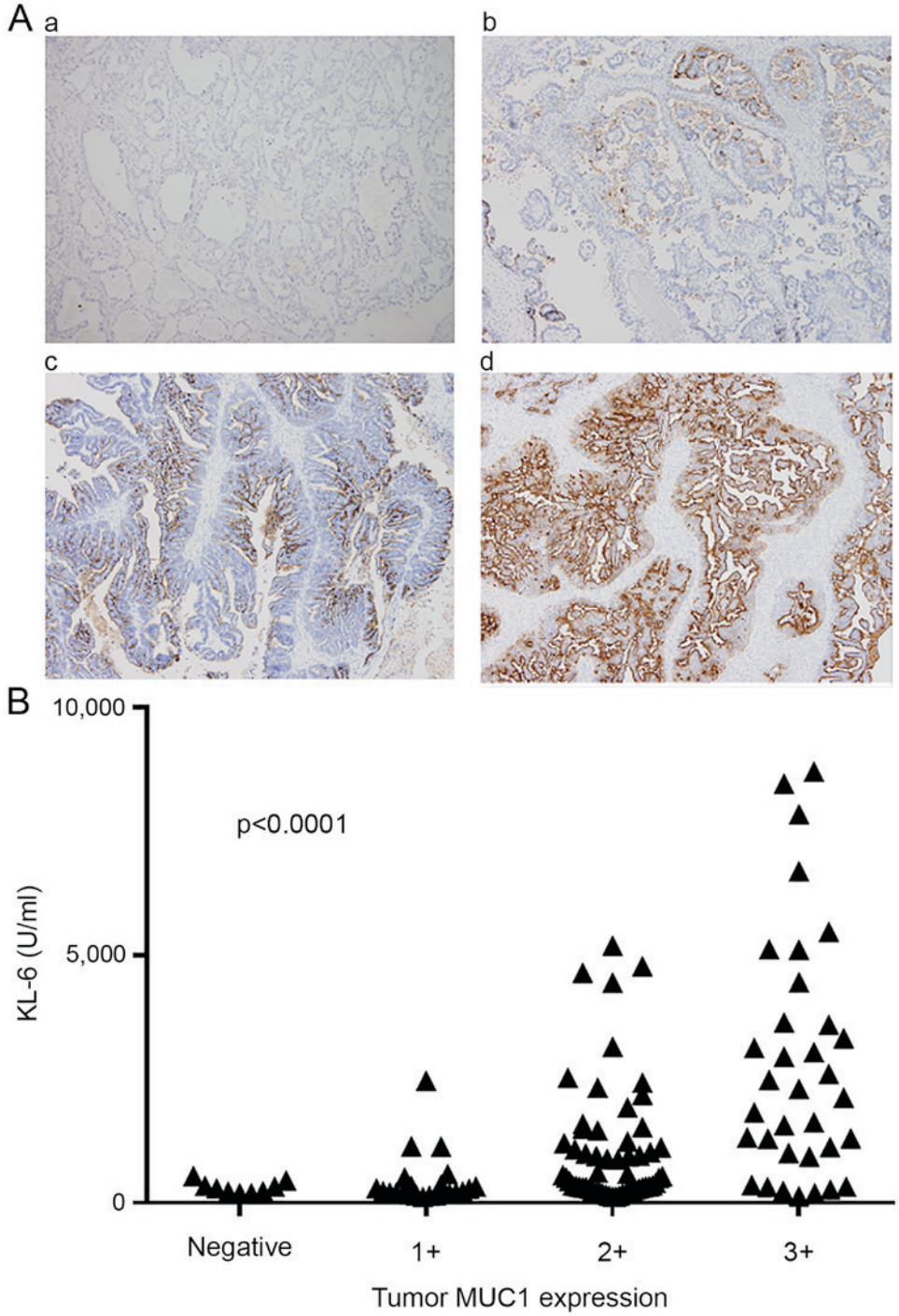

Figure 4. (A) Immunohistochemical staining of tumor MUC1 expression in ovarian cancers (magnification, 10x). Representative specimens are included for (a) negative staining, <1% positive staining; (b) 1+ staining, <25% positive staining; (c) 2+ staining, <75% positive staining; and (d) 3+ staining, ≥75% positive staining. (B) Association between serum KL-6 and tumor MUC1 expression. Serum KL-6 was determined using an electrochemiluminescent immunoassay, and tumor MUC1 expression was scored using immunohistochemical staining. Elevated serum KL-6 levels were frequently observed in tumors with 2+ and 3+ MUC1 expression. One-way analysis of variance and Tukey's post hoc analysis were used for the statistical analyses of differences in KL-6 level distribution between each group. KL-6, Krebs von den Lungen-6; MUC1, human mucin-1 glycoprotein.

reported that when serum MUC1 levels were measured using M2C5, a specific anti-MUC1 mAb, in 200 EOC patients and 396 healthy controls, the sensitivity and specificity for EOC were ~50 and 75%, respectively, at the optimum cut-off value.

KL-6 is a mucinous sialylated sugar chain on MUC1 (26). Serum KL-6 is elevated in the majority of patients with ILDs, and is widely accepted in Japan as a diagnostic test for ILDs and as a marker of disease activity (29,30). A number of studies have addressed circulating KL-6 in malignant tumors, including lung (43), breast (44) and hepatocellular carcinoma (45). However, to the best of our knowledge, no studies regarding serum KL-6 as a biomarker for EOC have been performed to date. In the present study, serum KL-6 in patients with EOC was retrospectively analyzed, and the results revealed that high serum KL-6 levels were correlated with disease progression. The ROC analysis

Table III. Summary of receiver operator characteristic curve analysis for predicting high tumor human mucin-1 glycoprotein expression using serum Krebs von den Lungen-6 levels.

Factor	Value
AUC	0.78
Youden's index	0.50
Cut-off value, U/ml	584
Sensitivity, %	59.1
Specificity, %	90.6
PPV, %	94.8
NPV, %	43.3
Accuracy, %	67.2

AUC, area under the curve, PPV, positive predictive value; NPV, negative predictive value.

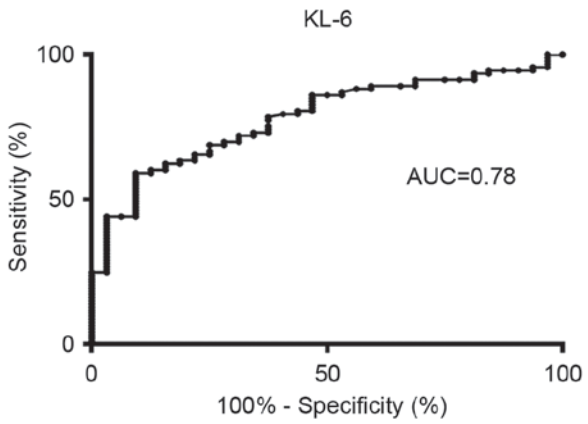

Figure 5. Serum KL-6 receiver operator characteristic curve to predict high tumor human mucin-1 glycoprotein expression as defined by an immunohistochemical staining score of 2+ and 3+. KL-6, Krebs von den Lungen-6; AUC, area under the curve.

data demonstrated that serum KL-6 levels, as a diagnostic marker, possess a higher sensitivity for EOC than CA125. Furthermore, the data suggest that serum KL-6 is able to distinguish stage I/II EOC from borderline and benign tumors. These results reinforce soluble MUC1 as a useful indicator of EOC, and demonstrate that serum KL-6 serves as a more effective diagnostic marker than CA125. A possible explanation may be that different anti-MUC1 mAbs bind to different epitopes of the MUC1 antigen.

The present study demonstrates serum KL-6 as prognostic marker for PFS in ovarian cancer, but not for OS. This may be associated with the treatment regimen employed following first relapse as different treatments may have been used between individuals, or reflect the response to frontline chemotherapy. It is also possible that the follow-up period was insufficient to make conclusions regarding OS. Budiu *et al* (46) reported serum MUC1 (CA15.3) level as a potential prognostic biomarker for platinum-resistant EOC, suggesting that high levels of soluble MUC1 predict a poor clinical response to chemotherapy.

Tumor MUC1 expression analyzed using immunohistochemistry was scored by the percentage of positively stained cells. The data of the present study demonstrated that serum KL-6 correlated with tumor MUC1 expression. The results indicate a potential for serum KL-6 measurements in assessing the local tumor MUC1 status non-invasively. Currently, >30 trials for MUC1-targeted therapeutics are being investigated in either early or late phase clinical trials, including the following: ImMucin, which is a 21mer synthetic vaccine composed of the entire signal peptide domain of the MUC1; tecemotide, which contains 25 amino acids from the variable number tandem repeat region of MUC1; ONT-10, a liposomal vaccine, which contains a unique tumor-specific antigen designed to mimic portions of MUC1; and GO-203, a cell-penetrating peptide-based inhibitor of MUC1 (15,47-49). Tumor heterogeneity is a pertinent obstacle in developing cancer therapeutics. Given that tumor characteristics are likely to differ in primary and relapsed tumors, serum KL-6 based assessments could aid in appropriate patient selection, thereby enabling progress in these MUC1-targeted therapies. Additionally, serum KL-6 levels may allow for real-time monitoring of tumor MUC1 expression during MUC1-targeted treatment. Our group is currently assessing the value of serum KL-6 in monitoring disease progression through a longitudinal study in cancer patients with recurrent ovarian cancer during standard chemotherapy.

One of the limitations for circulating KL-6 as a biomarker for EOC is its specificity for detecting ILDs. In the present study, 2 patients with benign ovarian tumors exhibited relatively high serum KL-6 levels. One patient was previously diagnosed with interstitial pneumonia and had serum KL-6 levels of 1,297 U/ml. This patient was the only one with ILD in the present study. The other patient, who had KL-6 levels of 1,091 U/ml, was a habitual heavy smoker, although it is unclear if smoking contributed to high serum KL-6 levels. Evidently, it may be difficult to interpret serum KL-6 levels in the diagnosis of EOC if the patients had ILDs as a comorbidity. Alternatively, patients with EOC may develop ILDs during the course of chemotherapy or targeted-therapy treatments, making it more complicated when judging the levels of serum KL-6. However, additional measurements of biomarkers for ILDs, such as surfactant proteins A and D (29) may aid in improving the understanding of more complicated cases.

In conclusion, serum KL-6 levels and tumor MUC1 expression were evaluated in patients with EOC in the present study. It was demonstrated that circulating KL-6 has a better diagnostic value in detecting EOC compared with CA125 in patients suspected of having malignant ovarian tumors. Furthermore, it was revealed that serum KL-6 may be complementary to CA125 at early stages of EOC detection. High levels of circulating KL-6 are associated with a shorter PFS than low levels of circulating KL-6 in patients with EOC. In addition, it was demonstrated that serum KL-6 correlates with tumor MUC1 expression, thus suggesting a potential application for MUC1-targeted therapy in patient selection and treatment monitoring.

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