Combined serum mesothelin and carcinoembryonic antigen measurement in the diagnosis of malignant mesothelioma

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Abstract. Malignant mesothelioma (MM) is a highly aggressive tumor associated with asbestos exposure. The identification of a marker specific for MM may be of considerable value for the early detection of this tumor and may be used in particular to screen groups with a history of asbestos exposure. The aim of this study was to evaluate serum soluble mesothelin-related peptide (SMRP) levels as a diagnostic marker for MM and investigate whether its diagnostic value is enhanced by combination with other biomarkers. Serum SMRP levels were measured using a quantitative enzyme-linked immunosorbent assay in 96 patients with MM, 55 patients with lung cancer and 39 individuals with a history of asbestos exposure. Receiver operating characteristic curves were constructed for performance evaluation. Stepwise logistic regression analysis was used to select marker combinations (MCs). Serum SMRP levels in patients with MM were significantly higher compared to those in the other groups ($P<0.001$). The sensitivity of SMRP levels in diagnosing MM was 56% and its specificity for MM vs. lung cancer and individuals with asbestos exposure was 87 and 92%, respectively. The area under the curve (AUC) was 0.76 [95% confidence interval (CI): 0.68-0.83] for the differentiation between MM and lung cancer and 0.78 (95% CI: 0.71-0.86) for the differentiation between MM and individuals with asbestos exposure. For the MC of presence of effusion, SMRP and carcinoembryonic antigen (CEA) levels, the AUC for the differentiation between MM and lung cancer (0.92; 95% CI: 0.88-0.97) and the differentiation between MM and individuals with asbestos exposure (0.93; 95% CI: 0.87-1.0) was significantly higher compared to that for SMRP alone ($P=0.0001$ and 0.0058, respectively). While the specificity of this MC was comparable to SMRP alone, its sensitivity was ~20% higher compared to that of SMRP alone. Therefore, combining SMRP and CEA improves the diagnostic performance of SMRP alone. A combination of serum biomarkers, including SMRP, may facilitate the non-invasive diagnosis of MM.

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Introduction

Malignant mesothelioma (MM) is a tumor that develops from the serous membranes that line the body cavities and it may arise in the pleura, peritoneum and pericardium; in addition, although extremely rare, it may also develop in the tunica vaginalis testis. The most common form of this disease is the malignant pleural mesothelioma (MPM). MM was previously considered as being extremely rare; however, its incidence and associated mortality rate exhibited a sharp increase worldwide over the last 50 years, due to the close association of MM with asbestos exposure. The prognosis of MPM is poor, with a median survival of ~9-17 months (1). However, in selected patients with epithelioid tumor histology, early-stage disease, who undergo trimodality treatment (combination of chemotherapy, postoperative radiotherapy and extrapleural pneumonectomy), median overall survival of 51 months and 5-year survival rates of 46% have been reported (2). Recent phase II trials reported a median survival of ~30 months for the patients who completed the trimodality treatment (3,4). Therefore, early diagnosis may play a vital role in the improvement of therapeutic outcomes. Together with the advances in imaging studies and endoscopic examinations, the development of biomarkers useful for serum or effusion diagnosis is crucial for the early diagnosis of MM. Currently known biomarkers for diagnosing MM include cytokeratin 19 fragment (CYFRA) (5-7), tissue polypeptide antigen (TPA) (5,6,8), hyaluronic acid (8), carbohydrate antigen (CA125) (8,9) and osteopontin (10-15). However, these markers have low specificity for MM.

Mesothelin is a 40-kDa cell surface glycoprotein that is overexpressed in cells of pancreatic and ovarian cancer, mesothelioma and other malignancies. The mesothelin gene encodes a 69-kDa glycoprotein, the mesothelin precursor protein, which is cleaved by a furin-like protease and its N-terminal region is released in the blood as a 31-kDa protein, the megakaryocyte

potentiating factor (MPF). The 40-kDa C-terminal region of this glycoprotein binds to the cell membrane as mesothelin. Three distinct variants of mesothelin have been identified, one of which has a modified C-terminus and becomes detached from the cell membrane since it lacks a glycosylphosphatidylinositol (GPI) anchor. This soluble isoform corresponds to the soluble mesothelin-related peptide (SMRP) (16). SMRP and MPF may be highly specific biomarkers for MM and have an equivalent diagnostic performance (17-19). SMRP is currently the most extensively investigated and is considered to be the best available blood protein biomarker of MM (20).

However, the diagnostic performance of SMRP alone is not considered to be sufficiently high, as it appears to exhibit insufficient sensitivity for MM (20,21). In diagnosing malignant tumors, such as ovarian or prostate cancer, the diagnostic performance of individual serum biomarkers was improved by combining data obtained using multiple biomarkers (22,23).

In the present study, we evaluated the performance of serum SMRP levels in the diagnosis of MM and investigated whether its diagnostic value could be improved through its combination with other biomarkers.

Materials and methods

Study design. The subjects of this study were patients who satisfied the following inclusion criteria: i) age ≥20 years; ii) pathologically proven MM or lung cancer; and iii) except for ii), individuals with asbestos exposure proven on the basis of their history or from the medical viewpoint. Only patients who personally provided written informed consent for the measurement of their serum biomarkers were enrolled in this study. Subjects who satisfied the above inclusion criteria during the study period were retrospectively enrolled. The pathological diagnosis was based on standard histological and immunohistochemical criteria (24,25). The subjects were classified into three groups: individuals with a history of asbestos exposure, patients with lung cancer and patients with MM. This study was approved by the Institutional Review Board of the Hyogo College of Medicine.

Measurement of serum biomarker levels. At the time of confirmation of the diagnosis, blood samples were collected from the subjects and, following prompt separation of the serum, the samples were stored at -80˚C. The serum SMRP levels were measured using an ELISA kit (Mesomark™; Fujirebio Diagnostics Inc., Malvern, PA, USA) according to the manufacturer's instructions. The serum levels of CYFRA and carcinoembryonic antigen (CEA) were measured using commercially available immunoassay systems according to the manufacturer's instructions: the serum CEA levels were determined using a chemiluminescent immunoassay (Abbott Japan Co., Ltd., Tokyo, Japan) and the serum levels of CYFRA were determined using a solid-phase sandwich immunoradiometric assay (CIS Bio International, Gif-sur-Yvette, France). The manufacturer suggests 3.5 ng/ml for CYFRA and 5.0 ng/ml for CEA as the cut-off values to differentiate between non-malignant disease and malignant tumors.

Statistical analysis. Summary statistics were used (median and 25th and 75th percentiles) to evaluate the distribution of serum SMRP levels. The Steel's test, a non-parametric form of the Dunnett's test, was used for comparing MM to the other groups. The sensitivity and specificity of SMRP for diagnosing MM were calculated, along with the corresponding 95% exact confidence intervals (CIs). The above analyses were also performed for CYFRA and its performance was compared to that of SMRP by using the McNemar's test. To compare the serum SMRP levels between each histological subtype of MM, the Steel-Dwass test, a non-parametric form of the Tukey's test, was performed. Subsequently, a stepwise logistic regression analysis was used to select marker combinations (MCs) that were more effective for diagnosing MM. The criterion for assessing whether a difference was significant in the variable selection was 5%. The diagnostic performance of SMRP and the MC was assessed by constructing a receiver operating characteristic (ROC) curve and calculating the area under the curve (AUC). The AUC for SMRP and that for the MC were compared using the theory on generalized U-statistics to generate an estimated covariance matrix and the χ^2 test (26). For each test, two-sided $P<0.05$ was considered to indicate a statistically significant difference. Data were analyzed using the statistical software SAS, version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and Stata, version 11.0 (StataCorp College Station, TX, USA). The GraphPad Prism software, version 4.00 for Windows (GraphPad Software, San Diego, CA, USA) was used to prepare the figures.

Results

Patient characteristics. A total of 190 subjects were enrolled in this study. A summary of the clinical characteristics of these subjects, together with a breakdown of each group by age, gender, history of asbestos exposure and presence of effusion (pleural or peritoneal) is presented in Table I. Among the 39 individuals with asbestos exposure, pleural plaque was present in 16, benign asbestos pleurisy in 7, asbestosis in 3 patients, asbestosis plus benign asbestos pleurisy in 5, round atelectasis in 2 and no imaging abnormalities in 6 patients. The histological subtype in the 55 patients with lung cancer was adenocarcinoma in 24, squamous cell carcinoma in 14 and small-cell carcinoma in 17 patients. Among the 96 patients with MM, the primary tumor site was the pleura in 91 and the peritoneum in 5 patients (Table II). The histological subtype was epithelioid in 57 patients, sarcomatoid in 12, biphasic in 6, desmoplastic in 4 and unspecified in the remaining 7 patients (Table II). Of the 91 patients with MPM, 74 were diagnosed with clinical stage IV disease according to the staging classification proposed by the International Mesothelioma Interest Group (IMIG). Only 5 patients had either stage I or II disease (Table II).

Performance of serum SMRP in diagnosing MM. Fujirebio Diagnostics, Inc., the developer of the Mesomark assay, recommends a cut-off value of 1.5 nM, which was the 99th percentile of the normal serum SMRP concentration in a population of 409 healthy Americans (27). An investigation in a population of healthy Germans revealed a cut-off value of 1.5-1.6 nM, which was the 95th percentile of the serum SMRP concentration (28). In our study, we performed a preliminary investigation of the distribution of serum SMRP levels among

Table I. Characteristics of the study subjects.

Characteristics	AE (n=39)	LC (n=55)	MM (n=96)
Age (years)			
Mean ± SD	68.1±8.1	64.7±10.6	61.2±9.5
Range	44-90	39-84	33-83
Gender			
Male	36	45	75
Female	3	10	21
Asbestos exposure			
Occupational	26	1	55
Environmental	13	1	27
None	0	53	14
Presence of effusion	12	16	78

AE, asbestos exposure; LC, lung cancer; MM, malignant mesothelioma; SD, standard deviation.

Table II. Demographic data of MM patients.

Characteristics	Patient no. (%)
Primary site	
Pleura	91 (94.8)
Peritoneum	5 (5.2)
Histological subtype	
Epithelioid	57 (59.4)
Sarcomatoid	12 (17.4)
Biphasic	16 (16.7)
Desmoplastic	4 (5.8)
NOS	7 (7.3)
Staging classification[a]	
I	3 (3.3)
II	2 (2.2)
III	12 (13.2)
IV	74 (81.3)

[a]Proposed by the International Mesothelioma Interest Group (IMIG), peritoneal mesothelioma (n=5) was excluded. MM, malignant mesothelioma; NOS, not otherwise specified.

72 healthy individuals without a history of asbestos exposure. Since this investigation revealed that 69 individuals (96%) had serum SMRP levels of <1.5 nM, we selected 1.5 nM, the 96th percentile, as the cut-off value.

The distributions of serum SMRP levels in each group are shown in Fig. 1. The serum SMRP levels in MM patients were significantly higher compared to those in the other groups (P<0.001) (Table III). The sensitivity of SMRP for diagnosing MM was 56% (95% CI: 46-66%) and its specificity for MM vs. lung cancer and individuals with asbestos exposure was 87% (95% CI: 76-95%) and 92% (95% CI: 79-98%), respectively (Table IV). By contrast, the sensitivity of CYFRA for diagnosing MM was 63% (95% CI: 52-72%) and its specificity for MM vs. lung cancer was 49% (95% CI: 35-63%) (Table IV). The sensitivity of SMRP and CYFRA did not differ significantly (P=0.157), although the specificity of SMRP for MM vs. lung cancer was significantly higher compared to that of CYFRA (P<0.001). The serum SMRP levels in epithelioid disease [median, 2.47 nM; interquartile range (IQR): 0.97-4.86] were significantly higher compared to those in sarcomatoid disease (median, 0.8 nM; IQR: 0.38-1.15) (P=0.04). However, there were no significant differences when compared to the other histological subtypes. There was no significant association between the serum SMRP levels and MPM stages (data not shown).

The diagnostic performance of SMRP was evaluated using ROC curves (Fig. 2). For the differentiation between MM and lung cancer, the AUC was 0.76 (95% CI: 0.68-0.83) (Fig. 2A) and for the differentiation between MM and individuals with asbestos exposure, the AUC was 0.78 (95% CI: 0.71-0.86) (Fig. 2B). For CYFRA, the AUC for the differentiation between MM and lung cancer was 0.55 (data not shown). Therefore, the diagnostic performance of SMRP for differentiating between MM and lung cancer was superior to that of CYFRA.

Investigation of MCs and their performance in diagnosing MM. To improve the performance of serum biomarkers in diagnosing MM, we investigated the optimal MCs. The measured variables common to patients with MM and lung cancer were age, gender, presence of effusion, clinical stage and the levels of SMRP, CYFRA and CEA. The measured variables common to patients with MM and individuals with a history of asbestos exposure were age, presence of effusion and the levels of SMRP, CYFRA and CEA. Since the distributions of all the biomarkers were significantly skewed to the right, the variables were logarithmically transformed using common logarithms. A stepwise logistic regression analysis was used to select the variables. To differentiate between MM and lung cancer, SMRP levels, presence of effusion and CEA levels were selected (Table V). From the signs of the estimates, we determined that the probability of a diagnosis of MM was higher for elevated SMRP levels, presence of pleural

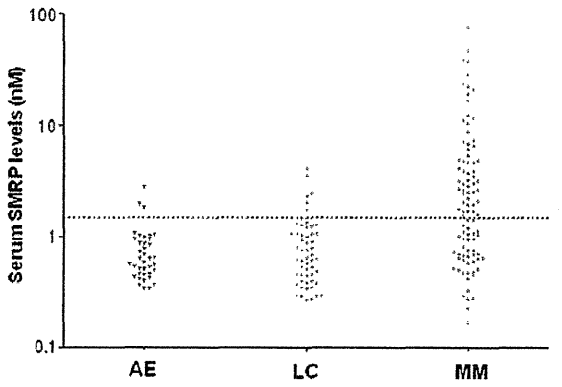

Figure 1. Distribution of serum soluble mesothelin-related peptide (SMRP) levels in each group. The serum SMRP levels in patients with malignant mesothelioma (MM) are compared to those in patients with lung cancer (LC) and individuals with a history of asbestos exposure (AE). The cut-off value is denoted by the horizontal dotted line.

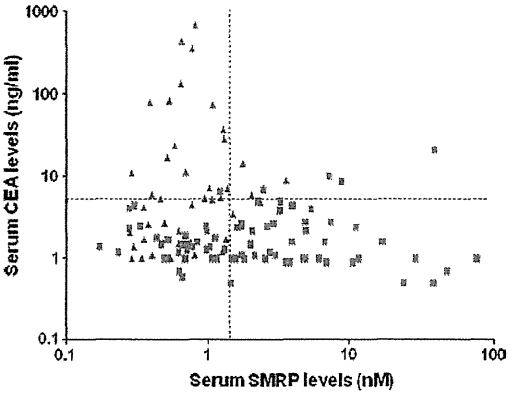

Figure 3. Scatter diagrams of serum biomarker levels in patients with malignant mesothelioma (■) and lung cancer (▲). Carcinoembryonic antigen (CEA) levels plotted against soluble mesothelin-related peptide (SMRP) levels. Each cut-off value is denoted by horizontal or vertical dotted lines.

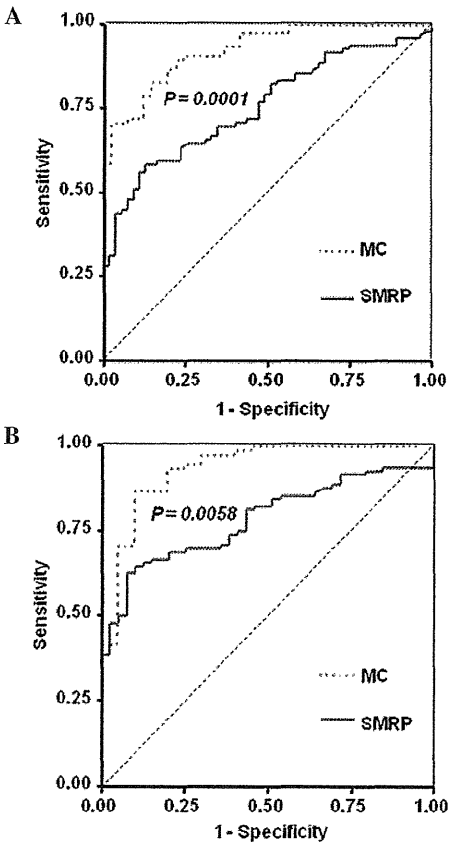

Figure 2. (A) Receiver operating characteristic (ROC) curves for soluble mesothelin-related peptide (SMRP) and the marker combination (MC) for differentiating between patients with malignant mesothelioma and lung cancer. The area under the curve (AUC) for the MC is significantly higher compared to that for SMRP alone (P=0.0001). (B) ROC curves for SMRP and the MC for differentiating between patients with malignant mesothelioma and individuals with a history of asbestos exposure. The AUC for the MC is significantly higher compared to that for SMRP alone (P=0.0058).

effusion and lower CEA levels. It was concluded that the selected markers were reasonable from the clinical standpoint. Subsequently, the markers selected to differentiate between MM and individuals with a history of asbestos exposure were age and CYFRA (data not shown). However, this model was composed of a single marker rather than multiple markers. Therefore, it was excluded from further investigation.

To further evaluate the models in Table V, the association between SMRP and CEA was analyzed using scatter diagrams (Fig. 3). The scatter diagrams demonstrated that the majority of patients with high CEA levels were those with lung cancer. In addition, the majority of patients with high SMRP levels were those with MM. Therefore, the combination of SMRP and CEA resulted in only a minor overlap of the diagnostic findings of MM and lung cancer, suggesting that the diagnostic performance for MM was improved. By contrast, since the combination of SMRP and CYFRA resulted in a significant overlap of the diagnostic findings of MM and lung cancer, it was inferred that the diagnostic performance was scarcely improved (data not shown).

The MC was composed using the results of Table V. Since the ratio of the estimates for SMRP, presence of effusion and CEA was ~3:1:5, the following MC was selected: MC=1xI(presence of effusion) + 3 x $\log_{10}$(SMRP) - 5 x $\log_{10}$(CEA), where I (presence of effusion) was defined as an indicator function with a value of 1 when effusion was present and 0 when effusion was absent. Wherein -1 was selected as the cut-off value to maximize the sum of the sensitivity and specificity, the sensitivity of MC for diagnosing MM was 76% (95% CI: 64-85%) and its specificity for MM vs. lung cancer and individuals with asbestos exposure was 88% (95% CI: 74-96%) and 90% (95% CI: 68-99%), respectively. While the specificity of MC was comparable to SMRP alone, its sensitivity was ~20% higher compared to that of SMRP alone. In addition, three of the five MPM patients with stage I-II disease were above the cut-off value, although none exhibited elevated serum levels of SMRP alone. The ROC curves for MC are shown in Fig. 2. The AUC for the differentiation between MM and lung cancer was 0.92 (95% CI: 0.88-0.97), which was significantly higher compared to that for SMRP alone (P=0.0001) (Fig. 2A). The AUC for the differentiation between MM and individuals with a history of asbestos exposure was 0.93 (95%CI: 0.87-1.0), which was also significantly higher compared to that for SMRP alone (P=0.0058) (Fig. 2B). These results indicate that combining CEA with SMRP improves the performance

Table III. Diagnostic findings based on the serum SMRP levels.

Serum SMRP levels (nM)	AE (n=39)	LC (n=55)	MM (n=96)
Mean ± SD	0.78±0.50	0.93±0.77	5.77±11.1
Median	0.64	0.65	1.88[a]
QR25-QR75	0.49-0.96	0.40-1.08	0.71-4.79
Min-max	0.30-2.80	0.30-4.10	0.30-75.4

[a]P<0.001, MM vs. AE or LC (by Steel's test). SMRP, soluble mesothelin-related peptide; AE, asbestos exposure; LC, lung cancer; MM, malignant mesothelioma; SD, standard deviation; QR25, 25th percentile; QR75, 75th percentile; min, minimum; max, maximum.

Table IV. Sensitivity and specificity of biomarkers for diagnosing MM.

Biomarkers	AE (n=39)	LC (n=55)	MM (n=96)
SMRP (%)			
Sensitivity	8	13	56
95% CI	2-21	5-24	46-66
Specificity	92	87	
95% CI	79-98	76-95	
CYFRA (%)			
Sensitivity	8	51	63
95% CI	2-21	37-65	52-72
Specificity	92	49	
95% CI	79-98	35-63	
CEA (%)			
Sensitivity	64	57	9
95% CI	41-83	41-72	4-17
Specificity	36	43	
95% CI	17-59	28-59	

MM, malignant mesothelioma; AE, asbestos exposure; LC, lung cancer; SMRP, soluble mesothelin-related peptide; CYFRA, cytokeratin 19 fragment; CEA, carcinoembryonic antigen; CI, confidence interval.

Table V. Results of stepwise logistic regression analysis (MM vs. LC).

Parameter	DF	Estimate	SE	Wald χ^2	P-value
Intercept	1	3.08	0.79	15.45	<0.001
SMRP[a]	1	2.83	0.92	9.48	0.002
Presence of effusion	1	1.28	0.42	9.15	0.003
CEA[a]	1	-5.52	1.46	14.20	<0.001

[a]The levels of SMRP and CEA were logarithmically transformed. MM, malignant mesothelioma; LC, lung cancer; DF, degree of freedom; SE, standard error of estimate; SMRP, soluble mesothelin-related peptide; CEA, carcinoembryonic antigen.

of SMRP alone in diagnosing MM and may facilitate early detection of MPM.

Discussion

The recent development of Mesomark, a quantitative ELISA kit using two monoclonal antibodies (OV569 and 4H3) that recognize SMRP, has enabled the measurement of serum SMRP levels. The findings of key studies on the performance of SMRP in diagnosing MM by using the Mesomark kit demonstrated that serum SMRP levels were significantly higher in MM patients compared to those in controls, such as healthy individuals, subjects with a history of asbestos exposure, or patients with asbestos-related benign pleural disease or lung cancer (9,11-21,27-35). In the present study, also undertaken using the Mesomark kit, the serum SMRP

levels were found to be significantly higher in MM patients compared to those in lung cancer patients and individuals with asbestos exposure. These findings are consistent with those first reported by Robinson *et al* (36), suggesting that the use of serum SMRP levels for diagnosing MM has excellent universality and reproducibility. Based on previous studies, including our own, SMRP is considered to be a highly specific biomarker for MM; however, its sensitivity, ranging from 48-80%, is moderate (9,11-21,27-35). To improve the performance of SMRP in diagnosing MM, there is a need to increase the sensitivity while maintaining a high degree of specificity.

One way of improving the sensitivity may be by lowering the cut-off value; however, this is not recommended, since it may result in a simultaneous reduction of specificity (26,28). Another approach may be to improve the diagnostic performance by combining data obtained using multiple biomarkers. The accuracy of the histopathological diagnosis of MM has markedly improved. One reason for this improvement has been the introduction of immunohistochemical analysis involving the combination of a positive marker that is highly expressed in MM and a negative marker that has a low frequency of expression in MM (37,38). A systemic review of markers for diagnosis of MM demonstrated that positive staining for CEA and epithelial antigen (clone Ber-EP4) and negative staining for epithelial membrane antigens and calretinin may confirm that a patient does not have MM (21). In addition, based on biomarker measurements in the pleural effusion, algorithms for the diagnosis of malignant pleural diseases were established. The CEA level achieved a greater accuracy in the differential diagnosis of MPM through its combination with other markers. For example, an elevated CYFRA level with a low CEA level in pleural effusion was shown to be highly suggestive of MPM (7).

To date, whether the combination of blood biomarkers, including SMRP, is able to improve the performance of SMRP alone in diagnosing MM remains controversial. A previous study by van den Heuvel *et al* (34) reported that the combination of two serum markers (CEA and SMRP) was the most accurate in differentiating MPM from non-small-cell lung cancer. The AUC of this marker combination demonstrated a significant improvement compared to the inverse levels of CEA alone. However, in that study, a direct comparison of diagnostic performance between this combination and SMRP alone was not performed.

Amati *et al* (31) evaluated the combination of two hematological biomarkers: 8-hydroxy-2'-deoxyguanosine (8-OHdG), an indicator of oxidative DNA damage and vascular endothelial growth factor β (VEGFβ), an angiogenic molecule. The results of that study indicated that the diagnostic performance of this combination in differentiating between healthy individuals and those with a history of asbestos exposure was superior to that of each biomarker alone. Although it was also mentioned that a combination of SMRP, 8-OhdG and VEGFβ was optimal for distinguishing between individual groups, including the MM group, that study provided no specific measures of diagnostic performance or any further details.

Several previous studies evaluated the diagnostic performance of combined SMRP and osteopontin measurements in MM. Creaney *et al* (12) demonstrated that the combination of SMRP, serum osteopontin and MPF did not exhibit increased sensitivity for detecting MM compared to that of SMRP alone. A recent study investigated serum SMRP and plasma osteopontin levels in 66 patients with MPM, 47 patients with non-malignant asbestos-related lung or pleural diseases, 42 patients with other benign pleural and lung diseases and 21 patients with lung cancer, as plasma osteopontin was proven to be more stable compared to serum osteopontin (14). A logistic regression analysis revealed that the combined marker model had an AUC of 0.912 and a sensitivity of 76%, with a 95% specificity (14). The AUC for this marker combination did not differ from that for serum SMRP alone. In previous studies, the majority of osteopontin-positive MM patients were also found to be positive for SMRP. This high degree of concordance may result in the finding that a combination of these two markers does not improve the performance of SMRP alone in diagnosing MM (12,14). Cristaudo *et al* (15) also measured serum SMRP and plasma osteopontin levels in 93 healthy subjects, 111 individuals with benign respiratory disease and 31 patients with MPM. That study was the first to demonstrate that a combination of these two markers was more efficient in MPM diagnosis compared to each marker used alone by means of the combined risk index, a new statistical approach of a logistic regression analysis. In that study, however, a small number of patients with MPM were enrolled and its histological subtype was limited to the epithelioid type. To confirm those findings, larger-scale studies are required. The combination of SMRP with CA125 (9), or MPF (12,18) has also been investigated. However, none of those studies demonstrated that the diagnostic performance of SMRP in combination with other markers outperformed that of SMRP alone.

The present study demonstrated that combining SMRP and CEA improved the diagnostic performance of SMRP alone, since these two markers act in a complementary manner. However, since we used the same data for selecting and assessing the performance of MC, it is possible that our evaluation of the MC may have been optimistic. Furthermore, in our study, data were collected from a single center; validation of the diagnostic performance of this particular MC by a multicenter study is recommended in the future.

It is difficult to determine whether pleural effusion developing in individuals with a history of asbestos exposure represents benign asbestos pleurisy or is an initial symptom of MPM and misdiagnosis at this stage may hinder the early detection of MPM. Future prospective research is required to confirm whether a combination of serum biomarkers, including SMRP, may be useful in diagnosing early-stage MPM.

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References

1. Tsao AS, Wistuba I, Roth JA and Kindler HL: Malignant pleural mesothelioma. J Clin Oncol 27: 2081-2090, 2009.
2. Sugarbaker DJ, Flores RM, Jaklitsch MT, *et al*: Resection margins, extrapleural nodal status, and cell type determine postoperative long-term survival in trimodality therapy of malignant pleural mesothelioma: results in 183 patients. J Thorac Cardiovasc Surg 117: 54-63, 1999.

3. Krug LM, Pass HI, Rusch VW, *et al*: Multicenter phase II trial of neoadjuvant pemetrexed plus cisplatin followed by extrapleural pneumonectomy and radiation for malignant pleural mesothelioma. J Clin Oncol 27: 3007-3013, 2009.
4. Van Schil PE, Baas P, Gaafar R, *et al*: Trimodality therapy for malignant pleural mesothelioma: results from an EORTC phase II multicentre trial. Eur Respir J 36: 1362-1369, 2010.
5. Bonfrer JM, Schouwink JH, Korse CM and Baas P: Cyfra 21-1 and TPA as markers in malignant mesothelioma. Anticancer Res 17: 2971-2973, 1997.
6. Schouwink J, Korse CM, Bonfrer JM, Hart AA and Baas P: Prognostic value of the serum tumour markers Cyfra 21-1 and tissue polypeptide antigen in malignant mesothelioma. Lung Cancer 25: 25-32, 1999.
7. Paganuzzi M, Onetto M, Marroni P, *et al*: Diagnostic value of CYFRA 21-1 tumor marker and CEA in pleural effusion due to mesothelioma. Chest 119: 1138-1142, 2001.
8. Hedman M, Arnberg H, Wernlund J, Riska H and Brodin O: Tissue polypeptide antigen (TPA), hyaluronan and CA 125 as serum markers in malignant mesothelioma. Anticancer Res 23: 531-536, 2003.
9. Creaney J, van Bruggen I, Hof M, *et al*: Combined CA125 and mesothelin levels for the diagnosis of malignant mesothelioma. Chest 132: 1239-1246, 2007.
10. Pass HI, Lott D, Lonardo F, *et al*: Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. N Engl J Med 353: 1564-1573, 2005.
11. Grigoriu BD, Scherpereel A, Devos P, *et al*: Utility of osteopontin and serum mesothelin in malignant pleural mesothelioma diagnosis and prognosis assessment. Clin Cancer Res 13: 2928-2935, 2007.
12. Creaney J, Yeoman D, Demelker Y, *et al*: Comparison of osteopontin, megakaryocyte potentiating factor, and mesothelin proteins as markers in the serum of patients with malignant mesothelioma. J Thorac Oncol 3: 851-857, 2008.
13. Rai AJ, Flores RM, Mathew A, *et al*: Soluble mesothelin related peptides (SMRP) and osteopontin as protein biomarkers for malignant mesothelioma: analytical validation of ELISA based assays and characterization at mRNA and protein levels. Clin Chem Lab Med 48: 271-278, 2010.
14. Creaney J, Yeoman D, Musk AW, de Klerk N, Skates SJ, and Robinson BW: Plasma versus serum levels of osteopontin and mesothelin in patients with malignant mesothelioma - which is best? Lung Cancer 74: 55-60, 2011.
15. Cristaudo A, Bonotti A, Simonini S, *et al*: Combined serum mesothelin and plasma osteopontin measurements in malignant pleural mesothelioma. J Thorac Oncol 6: 1587-1593, 2011.
16. Hassan R, Bera T and Pasten I: Mesothelin: a new target for immunotherapy. Clin Cancer Res 10: 3937-3942, 2004.
17. Ray M and Kindler HL: Malignant pleural mesothelioma: an update on biomarkers and treatment. Chest 136: 888-896, 2009.
18. Hollevoet K, Nackaerts K, Thimpont J, *et al*: Diagnostic performance of soluble mesothelin and megakaryocyte potentiating factor in mesothelioma. Am J Respir Crit Care Med 181: 620-625, 2010.
19. Cristaudo A, Bonotti A, Simonini S, Bruno R and Foddis R: Soluble markers for diagnosis of malignant pleural mesothelioma. Biomark Med 5: 261-273, 2011.
20. Hollevoet K, Reitsma JB, Creaney J, *et al*: Serum mesothelin for diagnosing malignant pleural mesothelioma: an individual patient data meta-analysis. J Clin Oncol 30: 1541-1549, 2012.
21. van der Bij S, Schaake E, Koffijberg H, Burgers JA, de Mol BA and Moons KG: Markers for the non-invasive diagnosis of mesothelioma: a systematic review. Br J Cancer 104: 1325-1333, 2011.
22. Schorge JO, Drake RD, Lee H, *et al*: Osteopontin as an adjunct to CA125 in determining recurrent ovarian cancer. Clin Cancer Res 10: 3474-3478, 2004.
23. Etzioni R, Falcon S, Gann PH, Kooperberg CL, Penson DF and Stampfer MJ: Prostate-specific antigen and free prostate-specific antigen in the early detection of prostate cancer: do combination tests improve detection? Cancer Epidemiol Biomarkers Prev 13: 1640-1645, 2004.
24. Travis WD, Brambilla E, Müller-Hermelink HK and Harris CC (eds.): World Health Organization Classification of Tumors, Pathology and Genetics. Tumours of the Lung, Pleura, Thymus, and Heart. IARC Press, Lyon, 2004.
25. Husain A, Colby T, Ordonez N, *et al*: Guidelines for pathologic diagnosis of malignant mesothelioma: a consensus statement from the International Mesothelioma Interest Group. Arch Pathol Lab Med 133: 1317-1331, 2009.
26. DeLong ER, DeLong DM and Clarke-Pearson DL: Comparing the areas under two or more correlated receiver operating characteristics curves: a nonparametric approach. Biometrics 44: 837-845, 1988.
27. Beyer HL, Geschwindt RD, Glover CL, *et al*: Mesomark: a potential test for malignant pleural mesothelioma. Clin Chem 53: 666-672, 2007.
28. Weber DG, Taeger D, Pesch B, Kraus T, Brüning T and Johnen G: Soluble mesothelin-related peptides (SMRP)-high stability of a potential tumor marker for mesothelioma. Cancer Biomark 3: 287-292, 2007.
29. Scherpereel A, Grigoriu B, Conti M, *et al*: Soluble mesothelin-related peptides in the diagnosis of malignant pleural mesothelioma. Am J Respir Crit Care Med 173: 1155-1160, 2006.
30. Cristaudo A, Foddis R, Vivaldi A, *et al*: Clinical significance of serum mesothelin in patients with mesothelioma and lung cancer. Clin Cancer Res 13: 5076-5081, 2007.
31. Amati M, Tomasetti M, Scartozzi M, *et al*: Profiling tumor-associated markers for early detection of malignant mesothelioma: an epidemiologic study. Cancer Epidemiol Biomarkers Prev 17: 163-170, 2008.
32. Pass HI, Wali A, Tang N, *et al*: Soluble mesothelin-related peptide level elevation in mesothelioma serum and pleural effusions. Ann Thorac Surg 85: 265-272, 2008.
33. Schneider J, Hoffmann H, Dienemann H, Herth FJ, Meister M and Muley T: Diagnostic and prognostic value of soluble mesothelin-related proteins in patients with malignant pleural mesothelioma in comparison with benign asbestosis and lung cancer. J Thorac Oncol 3: 1317-1324, 2008.
34. van den Heuvel MM, Korse CM, Bonfrer JM and Baas P: Non-invasive diagnosis of pleural malignancies: the role of tumour markers. Lung Cancer 59: 350-354, 2008.
35. Rodriguez Portal JA, Rodriguez Becerra E, Rodriguez Rodriguez D, *et al*: Serum levels of soluble mesothelin-related peptides in malignant and nonmalignant asbestos-related pleural disease: relation with past asbestos exposure. Cancer Epidemiol Biomarkers Prev 18: 646-650, 2009.
36. Robinson BW, Creaney J, Lake R, *et al*: Mesothelin-family proteins and diagnosis of mesothelioma. Lancet 362: 1612-1616, 2003.
37. Ordonez NG: The immunohistochemical diagnosis of mesothelioma: a comparative study of epithelioid mesothelioma and lung adenocarcinoma. Am J Surg Pathol 27: 1031-1051, 2003.
38. Ordonez NG: Application of mesothelin immunostaining in tumor diagnosis. Am J Surg Pathol 27: 1418-1428, 2003.

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Carboplatin plus Either Docetaxel or Paclitaxel for Japanese Patients with Advanced Non-small Cell Lung Cancer

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Abstract. *Aim: Assessment of the efficacy of docetaxel plus carboplatin vs. paclitaxel plus carboplatin in Japanese patients with advanced non-small cell lung cancer (NSCLC). Patients and Methods: Chemotherapy-naïve patients were randomly assigned at a ratio of 2 to 1 to receive six cycles of either docetaxel (60 mg/m^2) plus carboplatin [area under the curve (AUC)=6 mg/ml min] or paclitaxel (200 mg/m^2) plus carboplatin (same dose), on day 1 every 21 days. The primary end-point was progression-free survival (PFS). Results: A total of 90 patients were enrolled. Overall response rate, median PFS and median survival time in the docetaxel-plus-carboplatin group and the paclitaxel-plus-carboplatin group were 23% vs. 33%, 4.8 months vs. 5.1 months, and 17.6 months vs. 15.6 months, respectively. The docetaxel-plus-carboplatin group had a higher incidence of grade 3 or 4 neutropenia (88% vs. 60%). Conclusion: Both regimens were similarly effective in Japanese patients with advanced NSCLC.*

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Lung cancer is one of the most common malignancies and is the leading cause of cancer-related death worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 85% of all cases of lung cancer. Platinum-based chemotherapy has been considered a standard treatment for advanced NSCLC. In addition, molecular-targeted therapy, including vascular endothelial growth factor (VEGF) inhibitors such as bevacizumab, epidermal growth factor receptor (EGFR) inhibitors such as gefitinib or erlotinib, and anaplastic lymphoma kinase (ALK) inhibitors, has recently become a treatment option for specific subsets of patients, especially those with non-squamous cell lung cancer (2-5). These molecular targeted therapies have led to a paradigm shift of

treatment. Unfortunately, all patients with *EGFR*-mutant or ALK-positive lung cancer who receive EGFR or ALK inhibitors eventually experience disease relapse and require chemotherapy at some point during the course of treatment (4). Chemotherapy thus continues to play an important role in the management of NSCLC.

Docetaxel has been demonstrated to be effective against previously-untreated advanced NSCLC. Results of a large phase III trial found that docetaxel plus cisplatin was significantly superior to vindesine plus cisplatin in terms of overall response rate and overall survival (6). Carboplatin has shown broad equivalence to cisplatin in combination with chemotherapy for advanced NSCLC. To our knowledge, however, no clinical trial has directly compared docetaxel + carboplatin (DCarb) with paclitaxel plus carboplatin (PCarb) in patients with advanced NSCLC.

Fossella *et al.* reported a phase III study comparing docetaxel plus a platinum agent with vinorelbine plus cisplatin, performed by the TAX 326 Study Group (7). Docetaxel with cisplatin led to a better overall response and higher survival rate than docetaxel plus carboplatin, with a median survival time (MST) of 11.3 months, as compared with 9.4 months, respectively. However, that study was not designed to directly compare docetaxel plus cisplatin with docetaxel plus carboplatin. The therapeutic value of docetaxel with carboplatin as a front-line regimen for advanced NSCLC, thus remains unclear.

Millward *et al.* conducted a phase II study of docetaxel plus carboplatin in white and Asian patients with advanced NSCLC (8). The MST was 12.9 months, and multivariate analysis showed that ethnicity was a significant independent predictor of response and survival. Two clinical trials have evaluated docetaxel with carboplatin in Japanese patients with advanced NSCLC (9, 10). These trials reported a good MST of 12 months and 12.9 months, respectively. However, randomized phase II studies comparing docetaxel plus carboplatin with a standard regimen have yet to be performed on Asian patients with NSCLC. We therefore designed a randomized phase II study to compare the newer combination of DCarb with PCarb as standard treatment in patients with advanced NSCLC.

Patients and Methods

All patients enrolled in this study had cytologically- or histologically-confirmed diagnoses of NSCLC (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, or NSCLC not otherwise specified) with advanced stage IIIB or stage IV disease or relapse after surgical resection of NSCLC (regarded as stage IV). Other eligibility criteria were as follows: chemotherapy-naïve status; an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1; a neutrophil count of at least 2.0×10^9 cells/l; a platelet count higher than 100.0×10^9 cells/l; a hemoglobin concentration of at least 90 g/l; serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) concentrations of less than two-times the upper limit of normal (ULN); serum total bilirubin and creatinine concentrations of less than the ULN; a creatinine clearance of 50 ml/min or higher (as calculated by the Cockcroft-Gault equation) (11); and an alveolar partial pressure of oxygen (PaO_2) of 70 Torr or higher or an oxygen saturation on pulse oximetry (SpO_2) of 94% or higher (while breathing room air). Patients were excluded if they had any of the following conditions: severe infection, pregnancy or breastfeeding; a previous malignancy within the previous five years (except for patients with cured carcinoma *in situ*); another active cancer; an allergy to polysorbate 80 or polyoxyethylene castor oil; evidence of interstitial lung disease on a plain chest x-ray film; uncontrolled co-morbidities such as malignant hypertension, congestive heart failure, myocardial infarction within the previous six months, arrhythmia requiring treatment, bleeding tendency, or diabetes mellitus; pleural or pericardial effusion requiring drainage; symptomatic brain metastasis; or peripheral neuropathy of more than grade 1.

All patients provided written informed consent. The study protocol was approved by the Institutional Review Boards of all participating institutions and by the Japan Multinational Trial Organization (JMTO) ethical committee. This study was conducted in accordance with the Declaration of Helsinki and was registered with UMIN 000001225 on June 30, 2008.

Study design and treatment. This was a randomized, phase II, open-label study. The primary end-point was the determination of progression-free survival (PFS). The secondary end-points were tumor response, survival (1-year survival rate, overall survival), and toxic effects. Patients were randomly assigned at a ratio of 2 to 1 to receive either DCarbo or PCarbo. Central randomization to each arm was performed with the use of Pocock and Simon's method (12). Stratification factors were PS (0 or 1), more than 5% weight loss within the previous six months (yes or no), and serum lactic dehydrogenase (LDH) concentration (abnormally high or not).

Patients in the DCarbo group received intravenous docetaxel (60 mg/m^2) over the course of 60 to 90 min and carboplatin [area under the curve (AUC) 6 mg/ml min] over the course of three hours on day 1 every 21 days for six cycles. Pre-medication, such as anti-emetic agents or corticosteroids, was given as required. In the PCarbo group, patients received intravenous paclitaxel (200 mg/m^2) and carboplatin (AUC 6 mg/ml min, same as in the DCarbo group) on day 1 every 21 days for six cycles. Creatinine clearance was calculated using the Cockcroft-Gault equation. The serum creatinine level (mg/dl) used in this equation was modified by adding 0.2 mg/dl, because an enzyme assay is used in Japan, whereas Jaffe's non-enzyme assay was used to develop this equation. Patients in the PCarbo group were given pre-medication with dexamethasone, diphenhydramine, and ranitidine or cimetidine. The use of additional antiemetics was left at the physician's discretion. Use of granulocyte-colony stimulating factor (G-CSF) was permitted any time during the study (except for prophylactic use) in both groups. In the absence of progressive disease or intolerable toxicity, patients in both groups received six cycles of chemotherapy.

Treatment could be delayed for up to 14 days if the neutrophil count was less than 1.5×10^9 cells/l and the platelet count was less than 75×10^9 cells/l on day 1 of each course. In the event of prolonged or complicated grade 4 neutropenia or thrombocytopenia, the dose of docetaxel was reduced by 10 mg/m^2, that of paclitaxel by 25 mg/m^2, or that of carboplatin by AUC 1 mg/ml min for the subsequent cycle of chemotherapy. Dose reduction was allowed

twice. Treatment could be delayed for up to 14 days if AST or ALT (or both) was more than 2.5-times higher than the ULN, the serum creatinine concentration was more than 1.5-times higher than the institutional ULN, or nonhematological toxicity of grade 2 or higher developed (except for nausea, vomiting, fatigue, loss of appetite, mild electrolyte abnormalities, and alopecia) developed.

Patients were assessed every two cycles, and the objective response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0 (13). The best response in individual patients was derived from investigator-reported data. Objective response rates were confirmed by at least one sequential tumor assessment. Toxic effects were graded in accordance with the National Cancer Institute Common Toxicity Criteria, version 2.0 (14). The numbers and frequencies of each adverse event were respectively summarized for any grade and for grade 3 or higher in each treatment group. The MST with 95% confidence intervals (CI) and the probability of 1-year survival with 95% CI were calculated by the Kaplan-Meier method for each group.

Statistical plan and analysis. The primary end-point was PFS. The main objective of the study was to estimate the PFS rate at six months in the DCarbo group. The median PFS in the DCarbo group was predicted to be about 150 days on the basis of the results of previous studies. The PFS rate at six months was thus assumed to be 45%. Given that the range of the 90% CI at six months is 0.1 or less, we estimated that at least 60 patients would be required in the DCarbo group. Because patients were randomly assigned to either the DCarbo group or PCarbo group at a ratio of 2:1, the target number of patients in the latter group (calibration group) was 30. Hazard ratios (HR) and 95% CIs were calculated with a Cox proportional-hazards model.

Results

Patients' characteristics. A total of 90 patients were enrolled between June 2007 and September 2008 at 15 institutions in Japan. All patients were eligible for analysis. Sixty patients were assigned to the DCarbo group and 30 were assigned to the PCarbo group (Figure 1). The patients' characteristics for both groups were shown in Table I. The baseline characteristics of patients in the DCarbo group were similar to those in the PCarbo group.

Tumor response and survival. The total number of administered cycles of chemotherapy was 230 in the DCarbo group and 139 in the PCarbo group. The median follow-up time was 15.8 months.

Sixty patients began chemotherapy in the DCarbo group, and 19 completed six cycles according to protocol. The mean number of administered cycles of chemotherapy was 4.0 (range, 1 to 6). Dose modification was carried out once in 17 patients (28%) and more than once in 23 patients (38%). Treatment was delayed in 11 patients (18%). The reasons for treatment discontinuation before the completion of six cycles of DCarbo were disease progression (n=18), dose modification necessitated by adverse events more than twice (n=12), and withdrawal of treatment by the patient (n=6) or investigator (n=5). In the PCarbo group, 30 patients began chemotherapy, and 14 completed six cycles. The mean number of administered cycles was 4.6 (range, 1 to 6). Dose modification was carried out once in seven patients (23%) and more than once in seven patients (23%). Treatment was delayed in 10 patients (33%). The reasons for discontinuation of PCarbo before the completion of six cycles were disease progression (n=6), withdrawal of treatment by the patient (n=5), dose modification necessitated by adverse events more than twice (n=4), and withdrawal of treatment by the investigator (n=1).

The overall response rate (based on the best confirmed response during study treatment) was 23% [14 out of 60 patients with partial response (PR); 95% CI=13%-36%] in the DCarbo group and 33% (10 out of 30 patients with PR; 95% CI=17%-53%) in the PCarbo group (Table II). No patient had a complete response. Stable disease was obtained in 31 patients (52%; 95% CI=38%-65%) in the DCarbo group and 15 patients (50%; 95% CI=31%-69%) in the PCarbo group. The Median PFS was 4.8 months (95% CI=3.9-7.2 months) in the DCarbo group and 5.1 months (95% CI=4.4-6.4 months) in the PCarbo group. The PFS rate at six months was 42% (90% CI=31%-52%) in the DCarbo group and 40% (90% CI=25%-54%) in the PCarbo group (Figure 2). The hazard ratio of DCarbo referenced to PCarbo was 0.86 (95% CI=0.55-1.36). The MST was 17.6 months (95% CI=10.2-22.9 months) in the DCarbo group and 15.6 months (95% CI=9.3-20.8 monts) in the PCarbo group (Figure 3). The 1-year survival rate was 60% in both groups (90% CI=49%-70% in the DCarbo group and 44%-73% in the PCarbo group). The hazard ratio of DCarbo compared to PCarbo was 0.77 (95%CI=0.47-1.26).

Toxicity. All patients were assessable for toxicity (Table III). Patients in the DCarbo group had a higher incidence of grade 3 or 4 neutropenia than those in the PCarbo group (88% *vs*. 60%, 95% CI=77%-95% *vs*. 41%-77%). The PCarbo group had a higher incidence of grade 2 or more sensory neuropathy (37% *vs*. 3%, 95% CI=20%-56% *vs*. 0%-12%), myalgia (13% *vs*. 0%, 95% CI=4%-31% *vs*. 0%-6%), and arthralgia (20% *vs*. 2%, 95% CI=8%-39% *vs*. 0%-9%) than the DCarbo group. There were no major differences between the two groups regarding any other toxic effects (Table III).

One treatment-related death was reported in the DCarbo group. Acute respiratory distress syndrome (ARDS) developed in a 76-year-old woman two months after the end of the fifth, final cycle of treatment. Five days after the onset of respiratory failure, the patient had an acute myocardial infarction and died two days later. The patient's attending physician judged that the relation to treatment was "not definite." An independent data monitoring committee judged

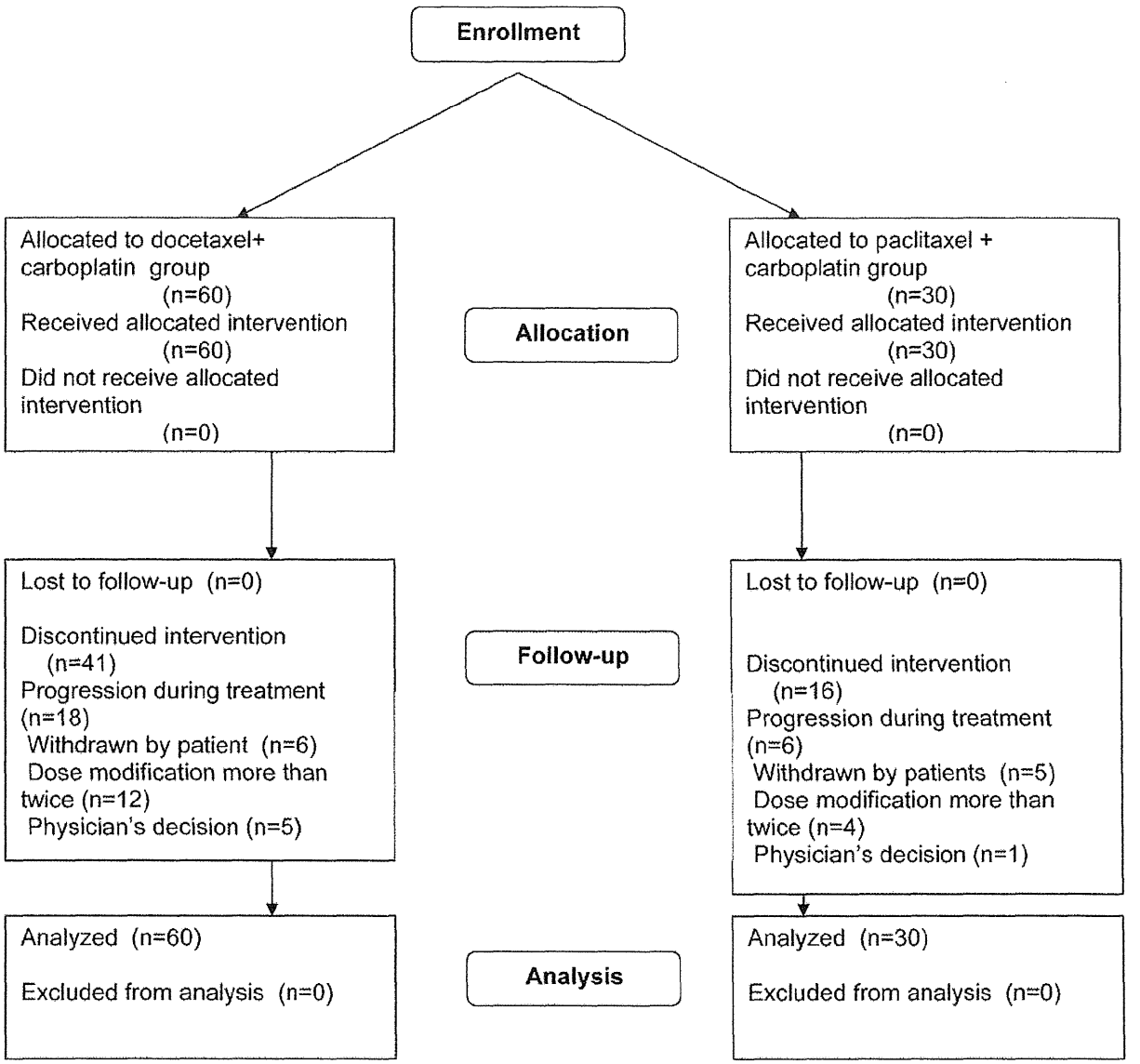

Figure 1. *Study design and patient flow. n: Number of patients.*

that the relation of death to the study treatment was not definite, but possible.

Discussion

This randomized phase II trial comparing DCarbo with PCarbo is the first of this kind to be performed in Asia. Our results suggest that both regimens are similar in terms of PFS and overall survival. The PFS of 4.8 (95% CI=3.9-7.2) months and MST of 17.6 (95% CI=10.2-22.9) months in the DCarbo group were favorable.

Asian ethnicity may contribute to some degree to better results in patients who receive DCarbo, as reported by Millward *et al.* (8). Three large phase III trials performed on Japanese patients with advanced NSCLC have included paclitaxel + carboplatin as one treatment arm (15-17). In these studies, the number of patients who received PCarbo was 281 (Okamoto *et al.*) (15), 197 (JMTO LC 00-03 study) (16), and 145 (Four-Arm Cooperative Study) (17), respectively. The dose of carboplatin was AUC 6 mg/ml min, with paclitaxel given at a dose of 200 mg/m^2 in two studies (15, 17) and 225 mg/m^2 in the other (16). The median PFS

Table I. *Patients' characteristics.*

	Docetaxel + carboplatin c (%) (n=60)	Paclitaxel + arboplatin (%) (n=30)
Age (median) (years)	67.5	65.5
Male/female	43/13 (78/22)	22/8 (73/27)
Body weight loss>5% Yes /no	11/49 (18/82)	5/25 (17/83)
Performance status 0/1	19/41 (32/68)	7/23 (23/77)
Histology Sq/Ad/La/Other	13/36/2/9 (22/60/3/15)	10/17/0/3 (33/57/0/10)
Stage IIIB/IV	24/36 (40/60)	10/20 (33/67)
Naïve/relapsed	53/7 (88/12)	26/4 (87/13)
LDH Normal/abnormally high	44/16 (73/27)	21/9 (70/30)
Prior radiotherapy	3 (5)	3 (10)

Sq: Squamous cell carcinoma, Ad: adenocarcinoma, La: large cell carcinoma, LDH: lactate dehydrogenase.

Table II. *Overall response and survival data.*

Regimen	Docetaxel + carboplatin	Paclitaxel + carboplatin
Number of patients	60	30
Response rate (95%CI)	23% (13-36%)	33% (17-53%)
Median PFS (95% CI), months	4.8 (3.9-7.2)	5.1 (4.4-6.4)
PFS rate (90% CI)*	42% (31-52)	40% (25-54)
HR (95% CI)	0.86 (0.55-1.36)	Referent
Median OS (95% CI), months	17.6 (10.2-23.0)	15.5 (9.4-20.8)
HR (95% CI)	0.77 (0.47-1.26)	Referent
1-Year survival rate (90% CI)	60% (49-70)	60% (44-73)

MST: Median survival time, CI: confidence interval, HR: hazard ratio, PFS: progression-free survival , OS: overall survival. *At six months.

Table III. *Toxicities experienced during study period.*

Toxicity	Docetaxel+ carboplatin % (95% CI) N=60	Paclitaxel+ carboplatin % (95% CI) N=30
Grade 3 or more Neutropenia	88 (77-95)	60 (41-77)
Grade 3 or more Anemia (hemoglobin)	12 (5-23)	7 (1-22)
Grade 3 or more Thrombocytopenia	0	3 (0-17)
Grade 3 or more Frbrile neutropenia	17 (8-29)	13 (4-31)
Grade 2 or more Nausea	28 (18-41)	17 (6-35)
Grade 2 or more Vomiting	12 (5-23)	10 (2-27)
Grade 2 or more Sensory neuropathy	3 (0-12)	37 (20-56)
Grade 2 or more Myalgia	0	13 (4-31)
Grade 2 or more Arthralgia	2 (0-9)	20 (8-39)
Possible TRD (ARDS)	1	0

CI: Confidence interval, TRD: treatment-related death, ARDS: acute respiratory distress syndrome.

or time to progression was 4.8, 5.8, and 4.5 months, and the MST was 13.3, 14.1, and 12.3 months, respectively. These results are similar to those of the present trial, obtaining a PFS of 5.1 months and an MST of 15.6 months, and suggest that Japanese patients have a good response to taxane-based chemotherapy. C1236T polymorphism in the ATP-binding cassette sub-family B member-1 (*ABCB1*) gene is significantly related to docetaxel clearance (18). Gandara *et al.* reported ethnic differences in the metabolism of taxanes between American and Japanese patients with lung cancer in a common-arm analysis of PCarbo, performed jointly in the United States and Japan (19).

Differences in the allelic distribution of genes involved in paclitaxel disposition or DNA repair [cytochrome *P450* 3A4 (CYP3A4)*1B and excision repair cross-complementation group 2 (*ERCC2*) K751Q] were observed between Japanese and American patients. Resulting metabolic differences in taxane metabolism may consequently contribute to better outcomes in Asian patients with lung cancer who receive taxanes.

In our study the dose of docetaxel was 60 mg/m^2 and that of carboplatin was AUC 6 mg/ml min. This dose of docetaxel is generally used in Japan to treat NSCLC. When combined with cisplatin, the dose of docetaxel used in Japan may be slightly lower the one that used in other countries (6). However, the results of Japanese studies in terms of PFS or overall survival are not inferior to those of studies performed in other countries, where docetaxel is usually given at a dose of 75 mg/m^2 (7). On the other hand, most Japanese studies have used cisplatin at a dose of 80 mg/m^2, which is slightly higher than that used in other countries (75 mg/m^2). The modest differences in the doses of chemotherapeutic agents may not have had a major influence on PFS or overall survival. Brunetto *et al.* reported that the dose intensity of platinum-doublet regimens including cisplatin or carboplatin with either vinorelbine or gemcitabine did not have an impact on survival or time-to-progression in patients with NSCLC (20).

A phase III study comparing DCarbo with PCarbo as first-line chemotherapy was performed in 1,077 patients with ovarian cancer (21). Docetaxel (75 mg/m^2) or paclitaxel (175 mg/m^2) with carboplatin to (AUC 5 mg/ml min) was administered every three weeks for six cycles.

The study also concluded that DCarbo is similar to PCarbo in terms of PFS and response, but recommended that longer follow-up is required before making a definitive statement on survival. DCarbo was considered an alternative first-line regimen for chemotherapy in patients with ovarian cancer. As for toxicity, DCarbo was associated with

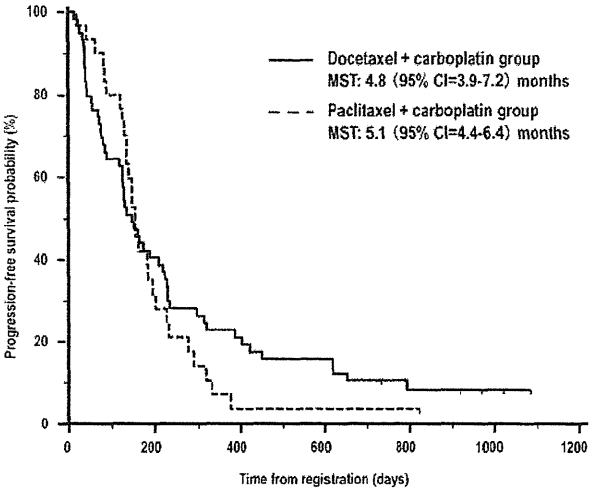

Figure 2. *Progression-free survival. MST: Median survival time, CI: confidence interval.*

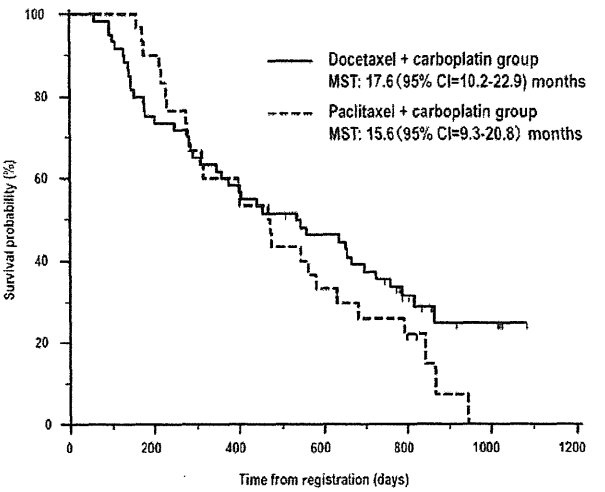

Figure 3. *Overall survival. MST: Median survival time, CI: confidence interval.*

substantially less overall and grade 2 or more neurotoxicity than PCarbo. On the other hand, DCarbo led to a higher incidence of grade 3 or 4 neutropenia than did PCarbo. Similar trends were noted in our study: DCarbo had a lower incidence of grade 2 or more sensory neuropathy (3% *vs.* 37%), but a higher incidence of grade 3 or more neutropenia (87% *vs.* 60%) as compared with PCarbo. Although myelosuppression was also frequently associated with DCarbo in our study, this adverse effect was not dose-limiting.

Recently, the survival of patients with NSCLC has improved, in part because of improved treatments or perhaps because of selection bias. The longer the survival, the more problematic is chronic toxicity such as neurotoxicity. Such toxicity negatively affects the quality of life of patients with NSCLC. This is especially true for those tested with PCarbo regimens (22). Even if the dose of paclitaxel is reduced from 225 mg/m^2 to 200 mg/m^2, the problem of neurotoxicity persists. DCarbo would, thus, be the preferred regimen to avoid severe neurotoxicity.

The treatment-related death in the DCarbo group in our study was reviewed by a safety committee. ARDS occurred as late as two months after the end of the patient's fifth, final cycle of treatment. The relation of death to chemotherapy with DCarbo was considered not definite, but possible.

Our study had several important limitations. We studied only Japanese patients, and it remains unclear whether our results can be extrapolated to other ethnic groups. Our study group comprised of patients with all histological types of NSCLC, and information on mutations in the *EGFR* gene was not obtained. In addition, the doses of docetaxel and carboplatin differed from those used in Western studies of patients with NSCLC.

Conclusion

Docetaxel plus carboplatin is considered an alternative first-line chemotherapeutic regimen for patients with newly-diagnosed advanced NSCLC, at least in Asia. In the future, this regimen might be combined with other treatments, such as molecular targeted therapy.

Conflicts of Interest

None.

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References

1 Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. CA Cancer J Clin *63*: 11-30, 2013.

2 Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, Seto T, Satouchi M, Tada H, Hirashima T, Asami K, Katakami N, Takada M, Yoshioka H, Shibata K, Kudoh S, Shimizu E, Saito H, Toyooka S, Nakagawa K, Fukuoka M, West Japan Oncology Group: Gefitinib *versus* cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): An open label, randomised phase III trial. Lancet Oncol *11*: 121-128, 2010.

3 Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, Fujita Y, Okinaga S, Hirano H, Yoshimori K, Harada T, Ogura T, Ando M, Miyazawa H, Tanaka T, Saijo Y, Hagiwara K, Morita S, Nukiwa T, North-East Japan Study Group: Gefitinib or chemotherapy for non-small-cell lung cancer with mutated *EGFR*. N Engl J Med *362*: 2380-2388, 2010.

4 Buettner R, Wolf J and Thomas RK: Lessons learned from lung cancer genomics: The emerging concept of individualized diagnostics and treatment. J Clin Oncol *31*: 1858-1865, 2013.

5 Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou SH, Dezube BJ, Janne PA, Costa DB, Varella-Garcia M, Kim WH, Lynch TJ, Fidias P, Stubbs H, Engelman JA, Sequist LV, Tan W, Gandhi L, Mino-Kenudson M, Wei GC, Shreeve SM, Ratain MJ, Settleman J, Christensen JG, Haber DA, Wilner K, Salgia R, Shapiro GI, Clark JW and Iafrate AJ: Anaplastic lymphoma kinase inhibition in non-small cell lung cancer. N Engl J Med *363*: 1693-1703, 2010.

6 Kubota K, Watanabe K, Kunitoh H, Noda K, Ichinose Y, Katakami N, Sugiura T, Kawahara M, Yokoyama A, Yokota S, Yoneda S, Matsui K, Kudo S, Shibuya M, Isobe T, Segawa Y, Nishiwaki Y, Ohashi Y and Niitani H: Phase III randomized trial of docetaxel plus cisplatin *versus* vindesine plus cisplatin in patients with stage IV non-small cell lung cancer: the Japanese Taxotere Lung Cancer Study Group. J Clin Oncol *22*: 254-261, 2004.

7 Fossella F, Pereira JR, von Pawel J, Pluzanska A, Gorbounova V, Kaukel E, Mattson KV, Ramlau R, Szczesna A, Fidias P, Millward M and Belani CP: Randomized, multinational, phase III study of docetaxel plus platinum combinations *versus* vinorelbine plus cisplatin for advanced non-small-cell lung cancer: The TAX 326 study group. J Clin Oncol *21*: 3016-3024, 2003.

8 Millward MJ, Boyer MJ, Lehnert M, Clarke S, Rischin D, Goh BC, Wong J, McNeil E and Bishop JF: Docetaxel and carboplatin is an active regimen in advanced non-small cell lung cancer: A phase II study in Caucasian and Asian patients. Ann Oncol *14*: 449-454, 2003.

9 Kasahara K, Kimura H, Shibata K, Araya T, Sone T, Oribe Y, Furusho S, Kita T, Shirasaki H, Yoshimi Y, Ueda A, Tachibana H, Shintani H, Mizuguchi M, Nishi K, Fujimura M and Nakao S: A phase II study of combination chemotherapy with docetaxel and carboplatin for patients with advanced or metastatic non-small cell lung cancer. Anticancer Res *26*: 3723-3728, 2006.

10 Kataoka K, Suzuki R, Taniguchi H, Noda Y, Shindoh J, Matsumoto S, Watanabe Y, Honda K, Suzuki K, Baba K, Imaizumi K, Kume H, Hasegawa Y and Takagi K: Phase I/II trial of docetaxel and carboplatin as a first-line therapy in patients with stage IV non-small cell lung cancer. Lung *184*: 133-139, 2006.

11 Cockcroft DW and Gault MH: Prediction of creatinine clearance from serum creatinine. Nephron *16*: 31-41, 1976.

12 Pocock SJ and Simon R: Sequential Treatment Assignment with Balancing for Prognostic Factors in the Controlled Clinical Trial. Biometrics *31*: 103-115, 1975.

13 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC and Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst *92*: 205-216, 2000.

14 NCI-CTC v2.0, 1999: http://ctep.cancer.gov/

15 Okamoto I, Yoshioka H, Morita S, Ando M, Takeda K, Seto T, Yamamoto N, Saka H, Asami K, Hirashima T, Kudoh S, Satouchi M, Ikeda N, Iwamoto Y, Sawa T, Miyazaki M, Tamura K, Kurata T, Fukuoka M and Nakagawa K: Phase III trial comparing oral S-1 plus carboplatin with paclitaxel plus carboplatin in chemotherapy-naive patients with advanced non-small cell lung cancer: Results of a West Japan Oncology Group study. J Clin Oncol *28*: 5240-5246, 2010.

16 Kubota K, Kawahara M, Ogawara M, Nishiwaki Y, Komuta K, Minato K, Fujita Y, Teramukai S, Fukushima M and Furuse K: Vinorelbine plus gemcitabine followed by docetaxel *versus* carboplatin plus paclitaxel in patients with advanced non-small cell lung cancer: A randomised, open-label, phase III study. Lancet Oncol *9*: 1135-1142, 2008.

17 Ohe Y, Ohashi Y, Kubota K, Tamura T, Nakagawa K, Negoro S, Nishiwaki Y, Saijo N, Ariyoshi Y and Fukuoka M: Randomized phase III study of cisplatin plus irinotecan *versus* carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small cell lung cancer: Four-Arm Cooperative Study in Japan. Ann Oncol *18*: 317-323, 2007.

18 Bosch TM, Huitema AD, Doodeman VD, Jansen R, Witteveen E, Smit WM, Jansen RL, van Herpen CM, Soesan M, Beijnen JH and Schellens JH: Pharmacogenetic screening of CYP3A and ABCB1 in relation to population pharmacokinetics of docetaxel. Clin Cancer Res *12*: 5786-5793, 2006.

19 Gandara DR, Kawaguchi T, Crowley J, Moon J, Furuse K, Kawahara M, Teramukai S, Ohe Y, Kubota K, Williamson SK, Gautschi O, Lenz HJ, McLeod HL, Lara PN Jr., Coltman CA, Jr., Fukuoka M, Saijo N, Fukushima M and Mack PC: Japanese-US common-arm analysis of paclitaxel plus carboplatin in advanced non-small cell lung cancer: A model for assessing population-related pharmacogenomics. J Clin Oncol *27*: 3540-3546, 2009.

20 Brunetto AT, Carden CP, Myerson J, Faria AL, Ashley S, Popat S and O'Brien ME: Modest reductions in dose intensity and drug-induced neutropenia have no major impact on survival of patients with non-small cell lung cancer treated with platinum-doublet chemotherapy. J Thorac Oncol *5*: 1397-1403, 2010.

21 Vasey PA: Role of docetaxel in the treatment of newly diagnosed advanced ovarian cancer. J Clin Oncol *21*: 136s-144s, 2003.

22 Rigas JR: Taxane-platinum combinations in advanced non-small cell lung cancer: A review. Oncologist *9(Suppl 2)*: 16-23m, 2004.

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Original Paper

Crosstalk between PI3 Kinase/PDK1/Akt/Rac1 and Ras/Raf/MEK/ERK Pathways Downstream PDGF Receptor

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Key Words
PDGF-ββ receptor • Akt • Rac1 • ERK • Crosstalk

Abstract
Background/Aims: Our earlier studies suggested crosstalk between IRS/PI3 kinase/PDK1/Akt/Rac1/ROCK and (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK pathways downstream PDGF-ββ receptor responsible for chemotaxis and proliferation of malignant mesothelioma cells. The present study was conducted to obtain evidence for this. ***Methods:*** To assess activation of Akt, MEK, and ERK, Western blotting was carried out on MSTO-211H malignant mesothelioma cells using antibodies against phospho-Thr308-Akt, phopho-Ser473-Akt, Akt, phospho-MEK, MEK, phopho-ERK1/2, and ERK1/2. To knock-down Akt, PI3 kinase, PDK1, and Rac1, siRNAs silencing each-targeted gene were constructed and transfected into cells. To monitor Rac1 activity, FRET monitoring was carried out on living and fixed cells. ***Results:*** ERK was activated under the basal conditions in MSTO-211H cells, and the activation was prevented by inhibitors for PI3 kinase, PDK1, Akt, and Rac1 or by knocking-down PI3 kinase, PDK1, Akt, and Rac1. Akt was also activated under the basal conditions, and the activation was suppressed by a MEK inhibitor and an ERK1/2 inhibitor. In the FRET analysis, Rac1 was activated under the basal conditions, and the activation was inhibited by a MEK inhibitor and an ERK1/2 inhibitor. ***Conclusion:*** The results of the present study show that ERK could be activated by PI3 kinase, PDK1, Akt, and Rac1 and that alternatively, Akt and Rac1 could be activated by MEK and ERK in MSTO-211H cells.

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Introduction

Platelet-derived growth factor (PDGF) promotes proliferation of malignant mesothelioma cells as well as other cancer types of cells [1-6]. The PDGF family includes PDGF-A, -B, -C and -D. PDGF-A and -B are secreted as active dimers composed of single-domain protein chains (PDGF-AA and -BB), but otherwise PDGF-C and -D, which contain an N-terminal CUB and a conserved C-terminal growth factor domain, are secreted as a latent dimeric factor and undergo proteolytic processing at the hinge region between the CUB domain and the growth factor domain to produce the active form of PDGF-CC and -DD through tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), respectively [7-9]. PDGF receptors consist of the PDGF-α and/or -β subunit such as αα homodimer, αβ heterodimer, and ββ homodimer [7]. PDGF-αα and -αβ receptors are activated by PDGF-AA, -BB, and -CC, and PDGF-ββ receptor is activated by PDGF-DD [8, 9].

In our earlier studies, PDGF-D, endogenously secreted, facilitated chemotaxis and promoted proliferation of malignant mesothelioma cells through PDGF-ββ receptor [10, 11]. PDGF-ββ receptor is a receptor tyrosine kinase, which phosphorylates the receptor by itself and insulin receptor substrate protein (IRS). IRS, in turn, recruits and activates phosphoinositide 3-kinase (PI3 kinase), to produce phosphatidylinositol (3,4,5)-trisphosphate [PI (3,4,5) P_3] from phosphatidylinositol (4,5)-biphosphate [PI (4,5) P_2]. PI (3,4,5) P_3 binds to and activates phosphoinositide-dependent kinase-1 (PDK1), which phosphorylates and activates Akt. Akt activates the Rho family Rac1/Cdc42, followed by activation of the effector Rho-associated coiled-coil forming protein kinase (ROCK). PDGF-ββ receptor, alternatively, phosphorylates Shc2, which forms a complex of Shc2/Grb2/SOS to activate Ras. Ras subsequently activates Raf followed by the sequent phosphorylation and activation of mitogen-activated protein (MAP) kinase cascades such as MAP kinase kinase (MEK) and extracellular signal-regulated kinase (ERK). Spontaneous proliferation of malignant mesothelioma cells was clearly suppressed by knocking-down PDGF-D and PDGF-ββ receptor [11]. The proliferation was significantly inhibited by the Akt inhibitor MK2206, and the ROCK inhibitor Y27632, but amazingly the PI3 kinase inhibitor wortmannin, the PDK1 inhibitor BX912, or the Rac1 inhibitor NSC23766 had no effect [11]. This suggests that Akt or ROCK is not activated along a PDGF-ββ receptor/PI3 kinase/PDK1/Akt/Rac1/ROCK axis. Moreover, proliferation of MSTO-211H cells was still inhibited by the MEK inhibitor PD98059 and the ERK1/2 inhibitor FR180204. Then, we hypothesized that crosstalk between IRS/PI3 kinase/PDK1/Akt/Rac1/ROCK and (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK pathways underlies downstream PDGF-ββ receptor in MSTO-211H cells.

To prove this hypothesis, the present study monitored activities of Akt and MEK/ERK by Western blotting and Rac1 using a plasmid encoded föerster resonance energy transfer (FRET) probe in MSTO-211H cells, a biphasic human malignant mesothelioma cell line. We show here that PI3 kinase, PDK1, Akt, and Rac1 could activate ERK in MSTO-211H cells and that MEK and ERK could otherwise activate Akt and Rac1.

Materials and Methods

Cell culture

MSTO-211H cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 0.003% (w/v) L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Western blotting

MSTO-211H cells were untreated and treated with a variety of inhibitors or transfected with small interfering RNAs (siRNAs), and then lysed in a lysate solution [150 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween-20 and 0.1% (w/v) sodium dodecyl sulfate (SDS), pH 7.5] containing 1% (v/v) protease inhibitor

cocktail and 1% (v/v) phosphatase inhibitor cocktail. The lysates were centrifuged at 3,000 rpm for 5 min at 4 °C. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a TGX gel (BioRad; Hercules, CA, USA) and then transferred to polyvinylidene difluoride (PVDF) membranes. Blotting membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently reacted with antibodies against phospho-Thr308-Akt (pT308)(Cell Signaling Technology, Inc.; Danvers, MA, USA), phospho-Ser473-Akt (pS473)(Cell Signaling Technology), Akt (Cell Signaling Technology), phospho-MEK (pMEK)(Cell Signaling Technology), MEK (Cell Signaling Technology), phospho-ERK (pERK)(Santa Cruz Biotechnology; Santa Cruz, CA, USA), ERK (Santa Cruz Biotechnology), PI3 kinase (Sigma-Aldrich; St. Louis, MO, USA), PDK1 (Sigma-Aldrich), Rac1 (Sigma-Aldrich), and β-actin (Sigma-Aldrich). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE healthcare; Piscataway, NJ, USA) and visualized using a chemiluminescence LAS-4000mini detection system (GE healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific; Waltham, MA, USA).

Construction and transfection of siRNA

The siRNA to silence the Akt1/2-targeted gene (Akt siRNA) was purchased from Santa Cruz Biotechnology. The siRNAs to silence the PI3 kinase p110δ-targeted gene (PI3K KD), the PDK1-targeted gene (PDK1 KD), the Rac1-targeted gene (Rac1 KD) were obtained from Ambion (Carlsbad, CA, USA). The sequences of siRNAs used were 5'-GUGAGAAAUUUGAACGGUUtt -3' and 5'-AACCGUUCAAAUUUCUCACta-3' for PI3 kinase p110δ; 5'-GGACACCAUCCGUUCAAUUtt-3' and 5'-AAUUGAACGGAUGGUGUCCtg-3' for PDK1; and 5'-CUACUGUCUUUGACAAUUAtt-3' and 5'-UAAUUGUCAAAGACAGUAGgg-3' for Rac1. Each negative control siRNA (NC siRNA)(Ambion) had the scrambled sequence, the same GC content, and nucleic acid composition. siRNAs were transfected into cells using a Lipofectamine reagent (Invitrogen; Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Monitoring of Rac1 activity

FRET probe containing Raichu-Rac1 with EV linker was kindly gifted from Dr. Matsuda (Kyoto university)[12, 13]. MSTO-211H cells were transfected with the FRET probe using an X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). Twenty-four h after transfection fluorescent signals in living cells were monitored at 485-nm argon laser for cyan fluorescent protein (CFP) and 517-nm argon laser for yellow fluorescent protein (YFP) in the presence and absence of inhibitors with a Zeiss LSM510 META inverted microscope (Oberkochen, Germany). In a different set of experiments, cells were untreated and treated with inhibitors for periods of time, and then fixed with formaldehyde [final conc. 3.7 (v/v) %] for 30 min. Then, CFP and YFP signals were monitored. The background was subtracted and the FRET ratio (YFP signal intensity/CFP signal intensity) was calculated using an ImageJ software (National Institutes of Health, USA).

Statistical analysis

Statistical analysis was carried out using unpaired *t*-test and Dunnett's test.

Results

PI3 kinase, PDK1, Akt, and Rac1 activates ERK

ERK was phosphorylated in the absence of inhibitors in MSTO-211H cells (Fig. 1A-E), indicating that ERK is spontaneously activated under the basal conditions. The PI3 kinase inhibitor wortmannin (10 μM) [14] reduced ERK phosphorylation (Fig. 1A). Expression of PI3 kinase was suppressed in MSTO-211H cells transfected with the PI3 kinase siRNA (Fig. 4A), confirming knocking-down PI3 kinase. ERK phosphorylation was significantly prevented by knocking-down PI3 kinase (Fig. 4C). It is indicated from these results that PI3 kinase has the potential to activate ERK.

The PDK1 inhibitor BX912 (100 nM) [15] significantly reduced ERK phosphorylation (Fig. 1B). Expression of PDK1 was suppressed in MSTO-211H cells transfected with the PDK1

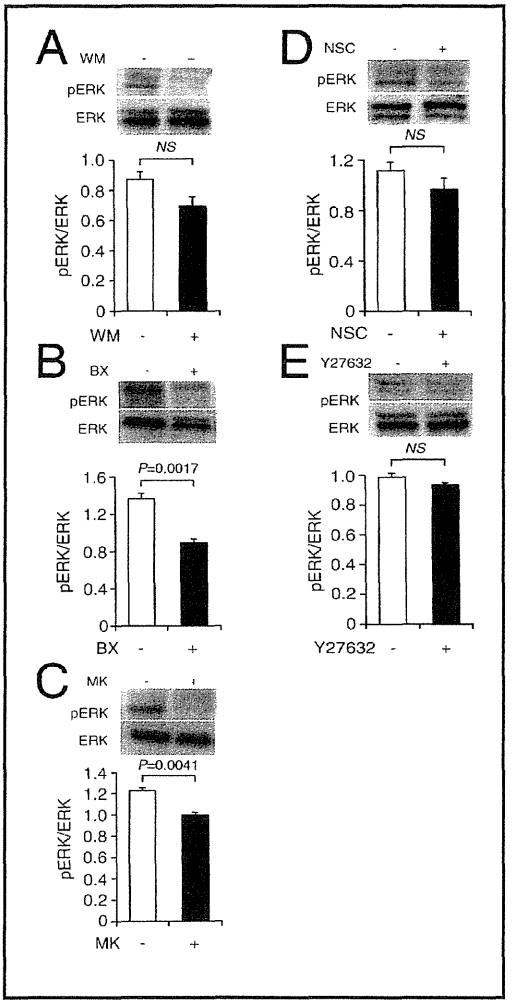

Fig. 1. ERK activation via a PI3 kinase/PDK1/Akt/Rac1 pathway. MSTO-211H cells were untreated (-) and treated (+) with wortmannin (WM)(10 μM)(A), BX912 (BX)(100 nM)(B), MK2206 (MK)(5 μM)(C), NSC23766 (NSC)(1 μM)(D), or Y27632 (10 μM)(E) for 10 min, and then, Western blotting was carried out using antibodies against pERK and ERK. In the graphs, each column represents the mean (± SEM) ratio of pERK signal intensity/ERK signal intensity (n=4 independent experiments). *P* values, Dunnett's test.

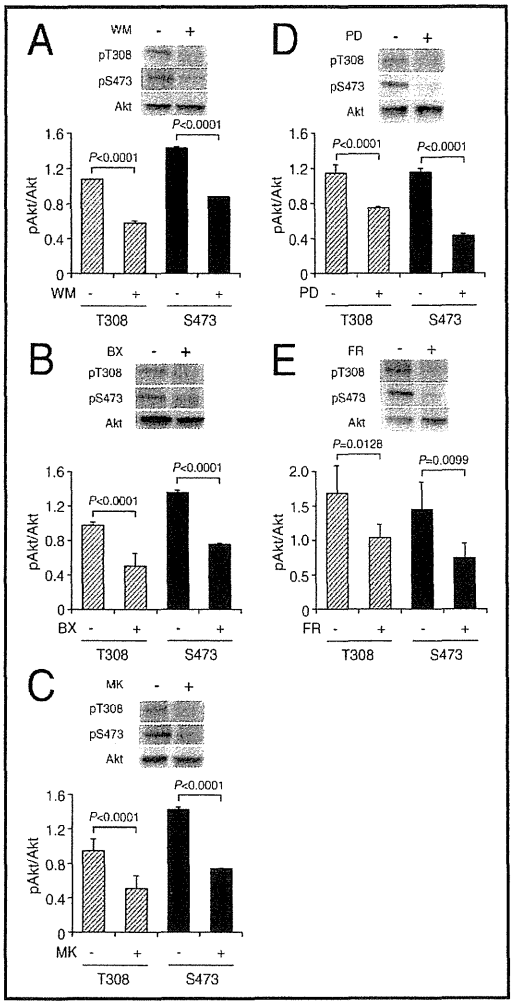

Fig. 2. Akt activation due to MEK and ERK. MSTO-211H cells were untreated (-) and treated (+) with wortmannin (WM)(10 μM)(A), BX912 (BX)(100 nM)(B), MK2206 (MK)(5 μM)(C), PD98059 (PD)(50 μM)(D), or FR180204 (FR)(10 μM)(E) for 10 min, and then, Western blotting was carried out using antibodies against pT308, pS473, and Akt. In the graphs, each column represents the mean (± SEM) ratio of pT308 signal intensity or pS473 signal intensity/Akt signal intensity (n=4 independent experiments). *P* values, Dunnett's test.

siRNA (Fig. 4D), confirming knocking-down PDK1. ERK phosphorylation was significantly prevented by knocking-down PDK1 (Fig. 4F). Collectively, these results indicate that PDK1 has the potential to activate ERK.

The Akt inhibitor MK2206 (5 μM) [16] significantly reduced ERK phosphorylation (Fig. 1C). Expression of Akt was significantly reduced in MSTO-211H cells transfected with the Akt siRNA (Fig. 3A), confirming Akt knocking-down. MEK phosphorylation was not attenuated by knocking-down Akt (Fig. 3B). Phosphorylation of ERK, on the other hand, was significantly

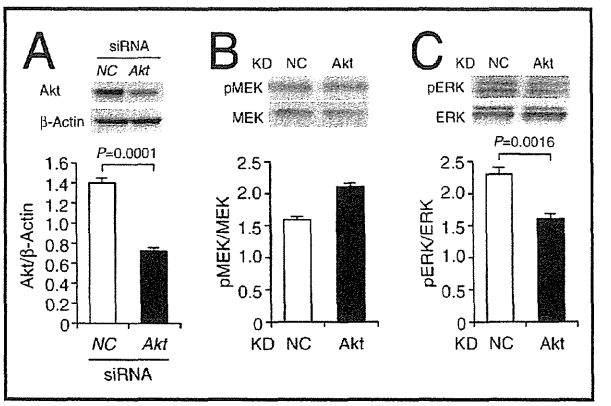

Fig. 3. Akt-mediated ERK activation. (A) Western blotting was carried out in MSTO-211H cells using antibodies against Akt and β-actin 48 h after transfection with the NC siRNA (NC) or the Akt siRNA. Signal intensities for Akt were normalized by those for β-actin. In the graph, each column represents the mean (± SEM) normalized expression of Akt (n=4 independent experiments). *P* value, unpaired *t*-test. In different sets of experiments, Western blotting was carried out using antibodies against pMEK and MEK (B) or pERK and ERK (C) 48 h after transfection with siRNAs. In the graphs, each column represents the mean (± SEM) ratio of pMEK signal intensity/MEK signal intensity or pERK signal intensity/ERK signal intensity (n=4 independent experiments). KD, knock-down. *P* value, Dunnett's test.

prevented by knocking-down Akt (Fig. 3C). These results interpret that Akt activates ERK directly, but not through MEK activation.

The Rac1 inhibitor NSC23766 (1 μM) [17] reduced ERK phosphorylation (Fig. 1D). Expression of Rac1 was suppressed in MSTO-211H cells transfected with the Rac1 siRNA (Fig. 4G), confirming knocking-down Rac1. ERK phosphorylation was significantly prevented by knocking-down Rac1 (Fig. 4I). These results indicate that Rac1 has the potential to activate ERK.

In contrast, the ROCK inhibitor Y27632 (10 μM) [18] had no effect on ERK phosphorylation (Fig. 1E), indicating no implication of ROCK in ERK activation. Taken together, these results imply that ERK could be still activated through a pathway along a PDGF-ββ receptor/PI3 kinase/PDK1/Akt/Rac1 axis.

MEK and ERK activate Akt and Rac1

Akt was phosphorylated at Thr308 and Ser473 in the absence of inhibitors in MSTO-211H cells (Fig. 2A-E), indicating that Akt is spontaneously activated under the basal conditions. Phosphorylation of Akt at Thr308 and Ser473 was inhibited by wortmannin (10 μM)(Fig. 2A) and BX912 (100 nM)(Fig. 2B). In addition, Akt phosphorylation at Thr308 and Ser473 was clearly inhibited by knocking-down PI3 kinase (Fig. 4B) and PDK1 (Fig. 4E). These results imply that Akt is activated through a pathway along a PDGF-ββ receptor/PI3 kinase/PDK1/Akt axis. Interestingly, Akt phosphorylation was also inhibited by MK2206 (5 μM)(Fig. 2C). This suggests that Akt might be activated through its autophosphorylation. Moreover, Akt phosphorylation was significantly inhibited by knocking-down Rac1 (Fig. 4H). This suggests that Rac1 is capable of activating Akt, i.e., Rac1 serves as a positive feedback activator of Akt.

Phosphorylation of Akt at Thr308 and Ser473 was also prevented by the MEK inhibitor PD98059 (50 μM) [19] (Fig. 2D) and the ERK1/2 inhibitor FR180204 (10 μM) [20, 21] (Fig. 2E). This indicates that MEK and ERK have the potential to activate Akt.

We finally monitored Rac1 activity in living and fixed MSTO-211H cells using a FRET probe. In the FRET analysis, increasing and reducing FRET ratio (YFP signal intensity/CFP signal intensity) correspond to activation and inactivation of Rac1, respectively. For living cells, the FRET ratio was apparently reduced by MK2206 (5 μM), while the ratio was not altered in the absence of inhibitors (Fig. 5A). This indicates that Rac1 is activated in an Akt-dependent manner under the basal conditions. The FRET ratio was also diminished by FR180204 (10 μM), but to a lesser extent than that for MK2206 (Fig. 5A), suggesting ERK-mediated Rac1 activation. For fixed cells, the FRET ratio was clearly inhibited by wortmannin

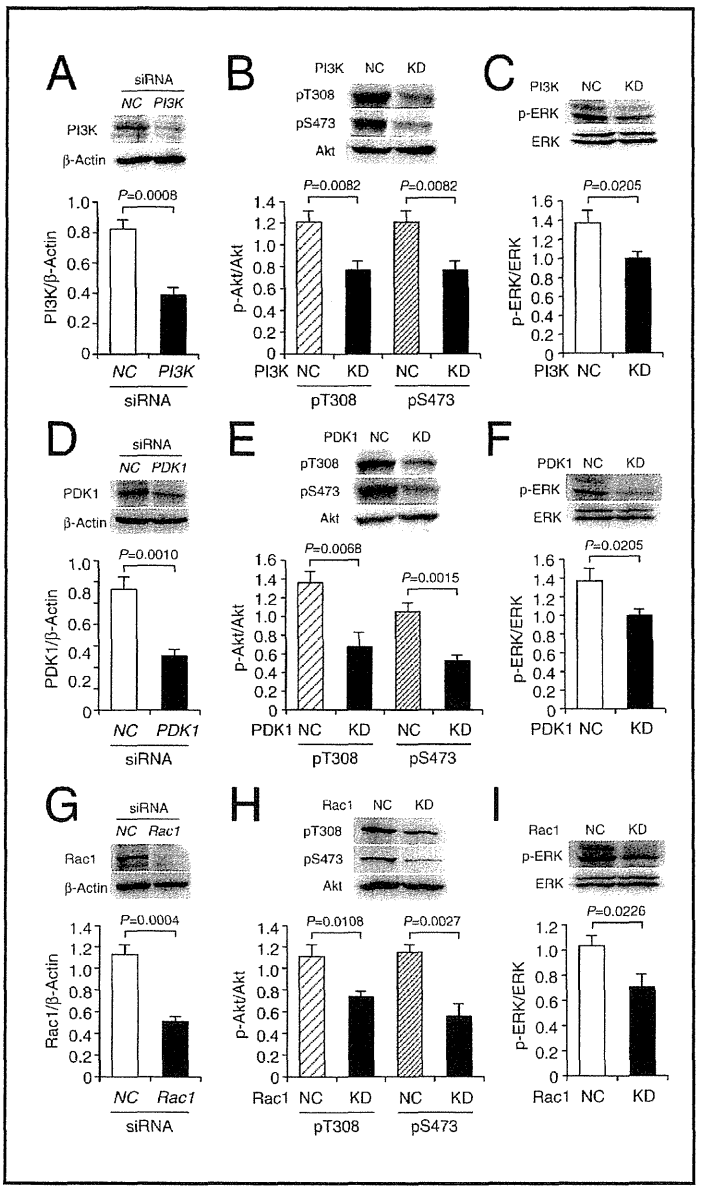

Fig. 4. ERK activation due to PI3 kinase, PDK1, and Rac1. Expression of PI3 kinase (A), PDK1 (D), and Rac1 (G) in MSTO-211H cells transfected with the NC and each siRNA. Signal intensities were normalized by those for β-actin. In the graphs, each column represents the mean (± SEM) normalized expression of PI3 kinase, PDK1, and Rac1 (n=4 independent experiments). *P* values, unpaired *t*-test. Akt activity in cells transfected with the NC siRNA, the PI3 kinase siRNA (B), the PDK1 siRNA (E), and the Rac1 siRNA (H). In the graphs, each column represents the mean (± SEM) ratio of pT308 signal intensity or pS473 signal intensity/Akt signal intensity (n=4 independent experiments). *P* values, Dunnett's test. ERK activation in cells transfected with the NC siRNA, the PI3 kinase siRNA (C), the PDK1 siRNA (F), and the Rac1 siRNA (I). In the graphs, each column represents the mean (± SEM) ratio of pERK signal intensity/ERK signal intensity (n=4 independent experiments). *P* values, Dunnett's test. PI3K, PI3 kinase; KD, knock-down.

(10 μM), BX912 (100 nM), and MK2206 (5 μM)(Fig. 5B), confirming Rac1 activation along a PI3 kinase/PDK1/Akt axis. The FRET ratio was still reduced by PD98059 (50 μM) and FR180204 (10 μM)(Fig. 5B). This indicates that Rac1 could be activated by MEK or ERK.

Discussion

PDGF-ββ receptor is implicated in two main pathways; an IRS/PI3 kinase/PDK1/Akt/Rac1/ROCK pathway and a (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK pathway (Fig. 6). In the present study, activation of Akt and ERK was found under the basal conditions in MSTO-211H biphasic human malignant mesothelioma cells. This, in the light of the fact that spontaneous proliferation of malignant mesothelioma cells was inhibited by knocking-down PDGF-D and PDGF-ββ receptor [11], indicates that Akt and ERK are activated via two main pathways linked to PDGF-ββ receptor, which is activated by PDGF-D endogenously secreted in MSTO-211H cells.