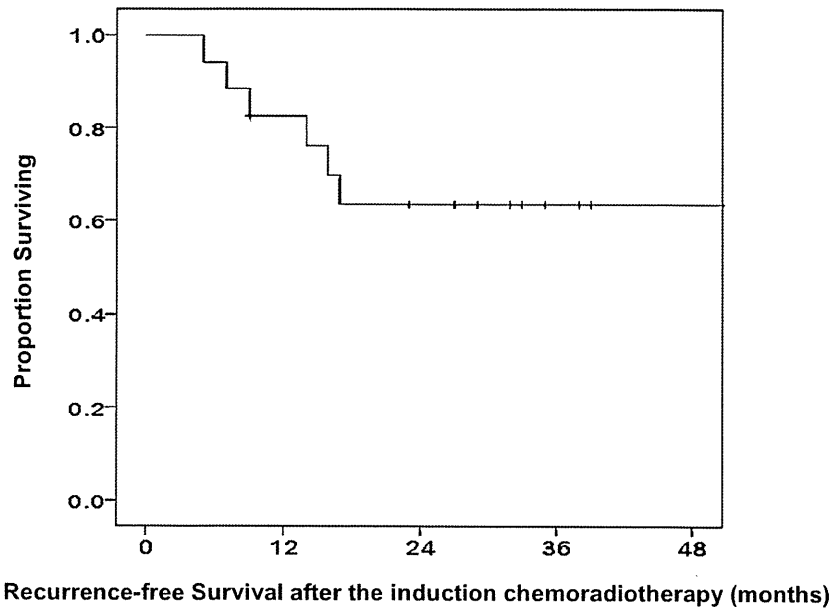

Fig. 1. Recurrence-free survival curve of patients who underwent a resection after receiving induction chemoradiotherapy

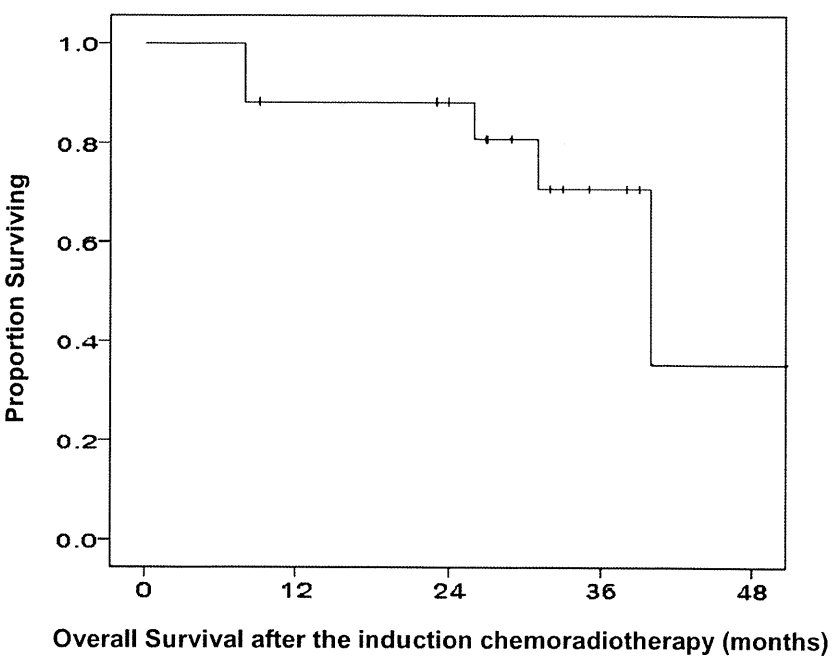

Fig. 2. Overall survival curve of patients who underwent a resection after receiving induction chemoradiotherapy

appears to be promising. A previous study using UFT plus cisplatin with concurrent radiotherapy as induction treatment for selected patients with stage IIIB NSCLC indicated that the 1- and 3-year survival rates in all patients were 73% and 56%, respectively.[6] The present study exceeds those results, without any severe toxicities. However, the response rate was 50% in the present study, which seems relatively low compared with other studies. Nevertheless the upper CI limit was over 70% in this study, so it would not necessarily be low if a larger number of patients were to be studied. A multi-institutional phase II trial must be conducted to establish the effectiveness of this induction treatment.

Surgery after chemoradiotherapy remains controversial for patients with stage IIIA-N2 NSCLC. Recently, Albain et al.[1] and van Meerbeeck et al.[2] reported the definitive data of two trials comparing chemotherapy and radiotherapy followed by surgical resection with

chemotherapy and radiotherapy for stage IIIA-N2 NSCLC. The first trial (INT 0139) used concurrent chemoradiotherapy as induction treatment. Four hundred and twenty-nine patients were randomly assigned at a 1:1 ratio to concurrent induction chemotherapy with cisplatin and etoposide plus radiotherapy (45 Gy). Patients in group 1 underwent a resection if there was no progression and those in group 2 continued radiotherapy uninterrupted up to 61 Gy. Two additional cycles of cisplatin and etoposide were given in both groups. The progression-free survival (PFS) in group 1 was better than in group 2, with a median of 12.8 versus 10.5 months (hazard ratio [HR] = 0.77, P = 0.017). However, the median OS was 23.6 months in group 1 versus 22.2 months in group 2 (HR = 0.87, P = 0.24).[1]

The latter study (EORTC 08941) was a randomized trial of radical surgery versus radiotherapy after a response to three cycles of platinum-based induction chemotherapy. Five hundred and eighty-two patients were registered in this study. The median and 5-year OS for patients randomly assigned to resection versus radiotherapy were 16.4 vs 17.5 months and 15.7% vs 14%, respectively (HR = 1.06, P = 0.6). The rates of PFS were also similar in both groups. Surgery therefore improved neither PFS nor OS in comparison with radiotherapy.[2]

Of interest, the INT 0139 trial found that deaths by the type of surgery were 1/98 (1%) for lobectomies and 14/54 (26%) for pneumonectomies, respectively.[1] An exploratory analysis determined that the median survival was improved for patients who underwent a lobectomy (33.6 vs 21.7 months, P = 0.002), but not for those who underwent a pneumonectomy (18.9 vs 29.4 months), versus chemotherapy plus radiotherapy without surgery. The investigators hypothesized that trimodality treatment could be beneficial if a complete resection with lobectomy can be done after chemotherapy plus radiotherapy, or if the mortality rate of pneumonectomy can be decreased.[1] An unplanned subgroup analysis of EORTC 08941 also found that patients who underwent a lobectomy or bilobectomy had better outcomes than those who had a pneumonectomy (5-year survival 27% vs 12%, respectively; P = 0.009).[2] A randomized phase III trial by the German Lung Cancer Cooperative group compared the intensity of the induction regimen: neoadjuvant concurrent chemoradiation versus chemotherapy alone.[13] Surgery was followed by radiotherapy in the latter group. The severe toxicity and mortality rates were significantly higher in the group of patients with preoperative chemoradiation, with almost double the postoperative mortality rate (9% vs 4.5%), especially after pneumonectomy. These three studies suggest that a pneumonectomy should therefore be avoided after induction chemoradiotherapy. However, Allen et al. reported that a pneumonectomy after chemoradiation could be safely accomplished in patients with advanced NSCLC in a single-institutional series. The 30- and 100-day mortality rates in their study were 6% and 10%, respectively. Their median survival was 23 months, with a 2-year survival rate of 49%.[14] There were neither deaths nor any major morbidities during the perioperative period in the present study, despite the fact that four complex pneumonectomies and three simple pneumonectomies were performed, confirming the findings by Allen et al.

Endobronchial ultrasound (EBUS)-guided transbronchial needle aspiration is emerging as an alternative to mediastinoscopy for mediastinal lymph node evaluation in NSCLC. However, for routine staging of the upper mediastinum in NSCLC, the benefits of EBUS over mediastinoscopy remain unproven. Therefore, mediastinoscopy is still the gold standard for mediastinal lymph node staging of NSCLC.[15] However, the procedure requires general anesthesia, and complications can occur. The new American College of Chest Physicians evidence-based practical guidelines for invasive staging of lung cancer were recently published.[16] Accordingly, invasive staging is not recommended for patients with extensive mediastinal infiltration (grade of recommendation: 2C). However, staging by CT or positron emission tomography scanning is still not sufficiently accurate, and invasive confirmation of the radiographic stage is recommended for patients with discrete node enlargement (grade of recommendation: 1B).

In conclusion, chemotherapy using S-1 plus cisplatin and concurrent radiotherapy as induction treatment followed by a surgical resection for selected patients with stage III NSCLC is feasible and appears to be a promising new treatment modality. A multi-institutional phase II trial is now being planned to establish the effectiveness of this induction treatment for patients with locally advanced NSCLC.

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Serum Heparan Sulfate Concentration is Correlated with the Failure of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Treatment in Patients with Lung Adenocarcinoma

Makoto Nishio, MD, PhD, Takeharu Yamanaka, PhD,† Kazuko Matsumoto, MD, PhD,‡ Hideharu Kimura, MD, PhD,‡ Kazuko Sakai, PhD,‡ Asao Sakai, MD, PhD,§ Takashi Sone, MD, PhD,§ Atsushi Horiike, MD,* Fumiaki Koizumi, MD, PhD,‖ Kazuo Kasahara, MD, PhD,§ Tatsuo Ohira, MD, PhD,¶ Norihiko Ikeda, MD, PhD,¶ Nagahiro Saijo, MD, PhD,# Tokuzo Arao, MD, PhD,‡ and Kazuto Nishio, MD, PhD‡*

Introduction: The epidermal growth factor receptor (*EGFR*) mutation status is a validated biomarker for the stratification of EGFR-tyrosine kinase inhibitor (EGFR-TKIs) treatment in patients with non-small cell lung cancer (NSCLC); however, its use is limited in patients with wild-type EGFR, and new biomarkers are needed. We hypothesized that the serum concentration of heparan sulfate (HS), which activates oncogenic growth factor receptor signaling through EGFR and non-EGFR signaling pathways, may be a novel glycobiological biomarker for EGFR-TKIs treatment in NSCLC.

Methods: The pretreatment serum HS concentrations were determined using enzyme-linked immunosorbent assay in 83 patients with stage IV non-small cell lung adenocarcinoma who received EGFR-TKIs treatment. The relationship between the serum HS concentrations and patient characteristics, tumor response, progression-free survival (PFS), and overall survival (OS) were analyzed.

Results: Patient sex, performance status, smoking history, and *EGFR* mutation status were associated with tumor response. The serum HS concentrations were significantly higher among patients with progressive disease than among those without progressive disease ($p = 0.003$). Furthermore, the serum HS concentrations were strongly associated with a poor PFS and OS in a univariate Cox analysis ($p = 0.0022$ and $p = 0.0003$, respectively). A stratified multivariate Cox model according to the *EGFR* mutation status showed that higher HS concentrations were significantly associated with a shorter PFS and OS ($p = 0.0012$ and $p = 0.0003$).

Conclusion: We concluded that a high-serum HS concentration was strongly related to a poor treatment outcome of EGFR-TKIs and may be a promising noninvasive and repeatable glycobiological biomarker in cancer treatment.

Key Words: Heparan sulfate, Non-small cell lung cancer, EGFR-tyrosine kinase inhibitors.

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Heparan sulfate proteoglycans (HSPGs) are composed of a core protein and one or more heparan sulfate (HS) glycosaminoglycan (GAG) chains. Many studies have demonstrated the importance of these molecules in development and normal physiology including metabolism, transport, information transfer, support, and regulation at the systemic level and the cellular level.[1] Heparin and HS consist of repeating disaccharide units that comprised a hexuronic acid and a D-glucosamine linked to each other and to other disaccharides by 1A4 linkages.[2] The HS component sugars (*N*-acetylgalactosamine and β-D-glucuronic acid/α-L-iduronic acid) and patterns of sulfating modifications create an extraordinarily large potential for structural diversity.[2] The structural diversity of HS is considered to be important because HS can bind and interact with a wide variety of proteins including thrombin, fibroblast growth factors (FGFs) 1 and 2, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin-8, MIP-1β, P-selectin, laminin, and fibronectin.[3] Such interactions are thought to mediate the enhancement of growth factor/receptor signaling activity, promote tumor growth, regulate differentiation, induce angiogenesis, modulate host immune cell responses to tumor cells, and promote metastasis in cancer cells.[3] Among the biological activities of HS, a large body of structural data

*Thoracic Oncology Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Koto-ku, Tokyo; †Institute for Clinical Research, National Kyushu Cancer Center, Minami-ku, Fukuoka; ‡Department of Genome Biology, Kinki University School of Medicine, Osaka-Sayama, Osaka; §Respiratory Medicine, School of Medicine, Kanazawa University, Kanazawa, Ishikawa; ‖Shien-Lab, National Cancer Center Hospital, Chuo-ku, Tokyo; ¶Department of Thoracic Surgery and Oncology, Tokyo Medical University, Shinjuku-ku, Tokyo; and #Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan.

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Address for correspondence: Tokuzo Arao, MD, PhD, and Kazuto Nishio, MD, PhD, Department of Genome Biology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan. E-mail: arao@med.kindai.ac.jp and knishio@med.kindai.ac.jp

M. Nishio, T. Yamanaka, and K. Matsumoto contributed equally to this work.

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has demonstrated that HS enhances FGFs/FGF receptor signaling by acting as a template that bridges FGF and the FGF receptor.[4] A structure-based proposal for an HS sequence able to bind FGF and FGFR showed that the interaction between HS and FGFs or FGFRs seemed to be determined by a specific sequence of 5-10 saccharides with sulfating modifications.[5] Other growth factor/receptor interactions may follow a similar binding and activation process. Thus, HS and HSPG expression may enhance the activity of oncogenic growth factor receptor signaling in cancer cells.

Meanwhile, selective epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) block EGFR signal transduction pathways implicated in the proliferation and survival of cancer cells[6–8] and have exhibited clinical activity against non-small cell lung carcinoma (NSCLC[9–11]). Several clinical and molecular biomarkers of EGFR-TKI treatment have been identified such as gender, smoking status, NSCLC histology, East Asian ethnicity, and an active *EGFR* mutation status that confers constitutively active tyrosine kinase activity and a hyperresponsiveness to gefitinib among patients with NSCLC.[12,13] These mutations are observed mostly in either point mutations in exons 18 (G719A/C) and 21 (L858R and L861Q) or in-frame deletions in exon 19 located at position 745.[14] Two recent phase III trials targeting adenocarcinoma in patients with NSCLC with *EGFR* mutations have demonstrated that the gefitinib group had a significantly longer progression-free survival (PFS) than the platinum-doublet therapy group.[15,16] These data indicated that the *EGFR* mutation status is a powerful predictor of the tumor response to EGFR-TKIs.

Recently, we have shown that the serum concentrations of heparin binding growth factors including heparin-binding EGF-like growth factor (HB-EGF), HGF, and VEGF are closely related to the treatment response of EGFR-TKIs in patients with NSCLC.[17] Our results have demonstrated that the serum concentrations of these growth factors were strongly related to the outcome of EGFR-TKIs treatment and suggest that these levels could be used to refine the selection of patients expected to respond to EGFR-TKIs treatment. On the basis of these findings, we speculated that the serum concentration of HS, which activates oncogenic growth factor receptor signaling through EGFR and non-EGFR signaling pathways, may be a novel glycobiological biomarker for EGFR-TKIs treatment in NSCLC. Identifying such a marker would contribute to the further individualization of treatment for NSCLC. In this report, we retrospectively studied the pretreatment serum HS concentrations in patients with stage IV non-small cell lung adenocarcinoma who underwent treatment with EGFR-TKIs.

PATIENTS AND METHODS

Patients

Pretreatment serum samples from histologically confirmed adenocarcinoma and patients with stage IV NSCLC (n = 93) were evaluated in this study. Six patients were excluded because their tumor response was not evaluated. Three additional patients were excluded because a complete clinical data set was not available, and a sufficient serum sample was not available for one patient. Thus, 83 patients were included in the final analysis. All the patients had been treated with EGFR-TKIs (gefitinib, n = 78; erlotinib, n = 5) at one of three centers (Kanazawa University, Japan; Cancer Institute Hospital, Japan; and Tokyo Medical University, Japan). The tumor response was evaluated every 2 to 3 months using computerized tomography according to the Response Evaluation Criteria in Solid Tumors; the response was then classified as a complete response, a partial response (PR), stable disease (SD), or progressive disease (PD). Clinicopathological features including age, sex, Eastern Cooperative Oncology Group performance status (PS), TNM stage, smoking status and *EGFR* mutation status were recorded. To detect active *EGFR* mutations, direct sequencing of a tumor sample was performed in 37 patients; 18 of these samples were found to harbor an *EGFR* mutation, whereas the remaining 19 samples exhibited wild-type EGFR. The mutation status of the other 46 patients was not evaluated. The median follow-up period was 8.2 months. This study was approved by the institutional review boards of all the centers involved in the study.

Preparation of Serum Samples

Blood samples were collected before the initiation of EGFR-TKI treatment. The separated serum was stocked at −80°C until use.

Serum HS Concentrations

Serum HS concentrations were determined using a human heparan sulfate enzyme-linked immunosorbent assay (ELISA) Kit (Code. No. 280564; Seikagaku Biobusiness, Tokyo, Japan). This sandwich-type ELISA kit is composed of two specific monoclonal antibodies recognizing the disaccharide units of HS. It specifically detects HS but does not crossreact with heparin, hyaluronic acid, chondroitin sulfate (CS), or keratin sulfate. In brief, a 50 μl aliquot of serum was treated with 5 μl of actinase E at a concentration of 20 mg/ml at 37°C for 20 hours; the reaction was stopped by heating at 100°C for 5 minutes. The sample was then centrifuged at 10,000 rpm for 10 minutes, and the supernatant (20 μl) was used for the analysis. The sample was diluted with 40 μl of the reaction buffer. Then, 20 μl of the samples were measured in duplicate according to the manufacturer's instructions. The absorbance of the samples at 450 nm and 630 nm was measured using VERSAmax (Japan Molecular Devices, Tokyo, Japan). The average was used for the subsequent analyses.

Statistical Analysis

The primary objective was to investigate novel markers correlated with treatment efficacy independently of EGFR status. If a molecule was very strongly associated with survival after adjustments for the EGFR status and important prognostic factors, then that molecule was deemed as warranting further prospective study to determine whether it was a predictive factor, a prognostic factor, or both. The distributions of the clinical factors were compared between patients with PD and those without PD using the Fisher's exact test. In terms of the analysis for survival time (PFS and overall survival [OS]), clinical factors including age, sex, Eastern Cooperative Oncology Group PS, and smoking status were

examined using the Cox proportional hazards model. After selecting the important clinical variables, we considered these variables fixedly in a Cox proportional hazards model and then determined whether the molecule was associated with survival independent of the important clinical variables at a two-sided significance level of 0.05. Log-transformed values were used for the molecule in the Cox models. The proportional hazards assumption was assessed graphically and using an individual time-dependent component for each covariate. In the multivariate Cox models, the EGFR status (wild type/mutant/unknown) was treated as a stratified variable. We applied the above analyses to all the cases, to the cases in which the EGFR status was evaluated, and to the cases with wild-type EGFR to check the robustness of the conclusions. The survival curves for PFS and OS were estimated using the Kaplan-Meier method. All the statistical analyses were performed using SAS for Windows (version 9.1.3).

RESULTS

Patient Characteristics and Tumor Response

The patient characteristics are listed in Table 1. All 83 patients were of Asian ethnicity and had been treated with EGFR-TKIs (gefitinib, $n = 79$; erlotinib, $n = 4$). Sixty-six (80%) and four (5%) patients had previously received chemotherapy and radiotherapy, respectively. Nineteen patients had wild-type EGFR, 18 had active mutations (exon 19, $n = 13$ and exon 21, $n = 5$), and 46 had an unknown status because their samples had been collected before the identification of this biomarker.[12,13] Regarding the response to EGFR-TKIs treatment, a PR was observed in 34 (41%) patients, SD was observed in 20 (24%) patients, and PD was observed in 29 (35%) patients; none of the patients exhibited a complete response. Significant differences in the tumor response were observed for patients characteristics such as a sex ($p = 0.0002$), PS ($p = 0.04$), smoking history ($p = 0.003$), and EGFR status ($p = 0.00001$). These findings were consistent with those of many previous reports.

TABLE 1. Patient Characteristics, Serum Concentration of Heparan Sulfate, and Response to EGFR-TKIs

		Response		
	Total ($n = 83$) (%)	PR + SD ($n = 54$)	PD ($n = 29$)	p
Age (yr)				0.65
≤65	43 (52)	27	16	
>65	40 (48)	27	13	
Sex				0.0002
Male	46 (55)	22	24	
Female	37 (45)	32	5	
PS				0.04
0–1	60 (72)	43	17	
2–4	23 (28)	11	12	
Smoking				0.003
Yes	51 (61)	27	24	
No	32 (39)	27	5	
CTx				0.27
Yes	66 (80)	41	25	
No	17 (20)	13	4	
RTx				0.08
Yes	4 (5)	1	3	
No	79 (95)	53	26	
EGFR status				0.00001[a]
Wild	19 (23)	6	13	
Mutant	18 (22)	18	0	
Unknown	46 (55)	30	16	—
HS				
Range	3.3–85.8	3.3–51.0	3.8–85.8	
Mean ± SD	14.9 ± 16.5	10.9 ± 9.8	22.2 ± 23.1	0.003

p values are calculated using the t test for serum concentration of heparan sulfate and the Fisher's exact test for other variables.

[a] Comparison between wild type and mutant.

HS, serum concentration of heparan sulfate (μg/ml); PR, partial response; SD, stable disease; PD, progressive disease; PS, performance status; EGFR-TKIs, epidermal growth factor receptor tyrosine kinase inhibitors; CTx, prior chemotherapy; RTx, prior radiotherapy; —, not done.

Serum Concentrations of HS and Tumor Response

The serum concentration of HS ranged from 3.3 to 85.8 μg/ml in all the patients (Table 1) and were over 20 μg/ml in 13 patients, indicating the presence of large individual differences in serum HS concentration. The serum HS concentration is shown for the tumor response groups in Figure 1. Of note, the serum HS concentration was significantly higher among patients with PD (22.2 ± 23.1 μg/ml) than among those without PD (10.9 ± 9.8 μg/ml, $p = 0.003$). The sensitivity and specificity of HS for discriminating PD from PR + SD were determined using the optimal cutoff value (13.5 μg /ml) obtained from a receiver operating characteristic (ROC) curve according to a previous report.[17] The sensitivity and specificity of HS for discriminating PD from PR + SD were 0.448 and 0.851, respectively.

Univariate Analysis of Clinical Molecular Factors for PFS and OS

The median PFS and OS were 4.1 and 10.2 months, respectively. Among the clinical factors that were examined, a male sex, a positive smoking history, and a poor PS were significantly related with a poor PFS and OS (Table 2). A higher serum HS concentration was significantly associated with a shorter PFS (HR, 3.61; $p = 0.0022$) and OS (HR, 5.57; $p = 0.0003$; Table 2). Thus, similar to the results for tumor response, a high serum HS concentration was closely associated with a poor EGFR-TKIs treatment outcome.

Figures 2*A*, *B* shows the Kaplan-Meier estimates for PFS and OS with respect to the concentrations of serum HS. All the patients were divided into two groups according to the cutoff value (13.5 μg /ml) described earlier. The curves indicated that the high serum HS group had a significantly poorer treatment outcome with respect to both PFS (median, 47 versus 161 days; $p = 0.002$) and OS (median, 105 versus 406 days; $p = 0.0002$).

Multivariate Analysis of Clinical Molecular Factors for PFS and OS

As the EGFR status of half the patients in this study was unknown, we used a stratified multivariate Cox analysis

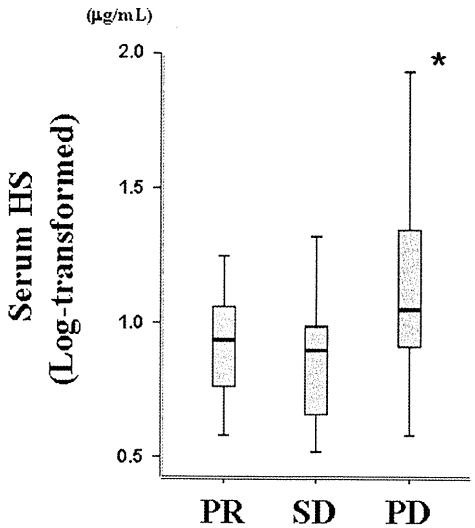

FIGURE 1. Box-whisker plots of serum HS concentration in patients with a partial response (PR, $n = 34$), stable disease (SD, $n = 20$), and progressive disease (PD, $n = 29$). HS, serum concentration of heparan sulfate (μg/ml). *Progressive disease (PD) versus PR + SD, $p < 0.05$.

TABLE 2. Univariate Analysis of Clinical and Molecular Factors for Progression-Free Survival and Overall Survival

	PFS			OS		
	Log Rank,	Cox		Log Rank,	Cox	
	p	HR	*p*	*p*	HR	*p*
Age (yr)						
>65 vs. ≤65	0.678	0.91	0.6791	0.978	0.99	0.9777
Sex						
Male vs. female	0.002	2.08	0.0026	<.0001	3.06	0.0001
Smoke						
Yes vs. no	0.024	1.74	0.0257	0.011	1.99	0.013
PS						
2–4 vs. 0–1	0.075	1.56	0.079	0.002	2.31	0.0021
HS						
Continuous	n.d.	3.61	0.0022	n.d.	5.57	0.0003

Univariate analyses of factors for progression-free survival (PFS) and overall survival (OS) were for all the patients. Log-transformed values were used for all the molecules.

HR, hazard ratio; n.d. not done; HS, serum concentration of heparan sulfate (μg/ml); PS, performance status.

that included the EGFR status as a stratification factor[18] (Table 3). First, sex and PS remained statistically significant at a level of 0.05 in a multivariate model after backward selection. The smoking status was no longer significant ($p = 0.46$ and $p = 0.40$ for PFS and OS, respectively) because it was highly correlated with sex ($p < 0.0001$, Fisher's exact test). Thus, we used sex and PS as fixed factors in the Cox model. The serum HS concentration was significantly correlated with poor treatment outcomes for PFS (HR = 3.98, $p = 0.0012$) and OS (HR = 5.42, $p = 0.0003$) in the final model; a high concentration of HS was correlated with a shorter PFS and OS independently of the EGFR status, sex, and PS (Table 3). In the final model, no interaction was shown between the EGFR status and the serum HS concentration ($p > 0.20$ for both PFS and OS). The results presented in Table 3 were also stable in analyses of subsets of patients with a known EGFR status as well as patients with wild-type EGFR (data not shown). Regarding *EGFR* mutations and the HS concentration, we verified the results in additional experiments using an independent set of 48 serum samples from patients whose tumor EGFR status was known. The results showed that a high serum HS level was reproducibly associated with a poor PFS during EGFR-TKI treatment, although the p value was not significant ($p = 0.087$, Supplementary Figure 1A, http://links.lww.com/JTO/A109). The EGFR status did not seem to be associated with the serum HS concentrations in the 48 additional samples ($p = 0.48$, Supplementary Figure 1B, http://links.lww.com/JTO/A109).

Taken together, these observations suggested that a high serum HS concentration was significantly associated with the failure of EGFR-TKIs treatment and may be a novel glycobiological biomarker.

DISCUSSION

The major GAG in the blood is CS, and other serum GAGs include HS, keratin sulfate, and hyaluronan.[19] Many methods are now available to measure the concentration of serum/plasma GAGs; these methods include cellulose acetate membrane electrophoresis, paper, affinity, and gas chromatography, capillary electrophoretic analysis, and HPLC. Nevertheless, no standardized methods exist for serum/plasma GAG isolation and quantification.[19] Our approach using a sandwich ELISA was easy to perform, quantitative, and reproducible. The C.V. value was below 10% in intraplate, intrakit, and intraday analyses (data of Seikagaku-kogyo). Regarding individual differences, the Alcian blue dot blot method showed that the GAG concentration of plasma from hospitalized patients exhibited a variation of plasma GAGs of 0.1 to 17.6 μg/ml,[20] and our result for the HS concentration was 3.3 to 85.8 μg/ml. Identifying the cause of these individual differences will require further study. Recent studies have shown that the pleural fluid/serum GAG ratio may be useful for the simultaneous differentiation of exudates from transudates and of malignant exudates from benign exudates.[21] In ovarian cancer, the serum CS level may be useful as a discriminator between benign ovarian disorders and malignant ovarian diseases.[22]

Accumulating evidence has demonstrated that HSPG has oncogenic roles in cancer cells. Perlecan is a potent inducer of bFGF-mediated neovascularization in vivo.[23] In addition, several studies have shown that large deposits of perlecan were observed in the tumor stroma and blood vessel walls in liver tumor and invasive breast cancer in clinical specimens.[24,25] The strong reactivity for perlecan in tumoral stromal vessels suggests a role for these HSPGs in tumoral angiogenesis, and the angiogenic effect is considered to interact with various proangiogenic ligands.[26] On the other hand, high expression levels of shed/soluble syndecans-1 are found in the serum of patients with myeloma and lung cancer,

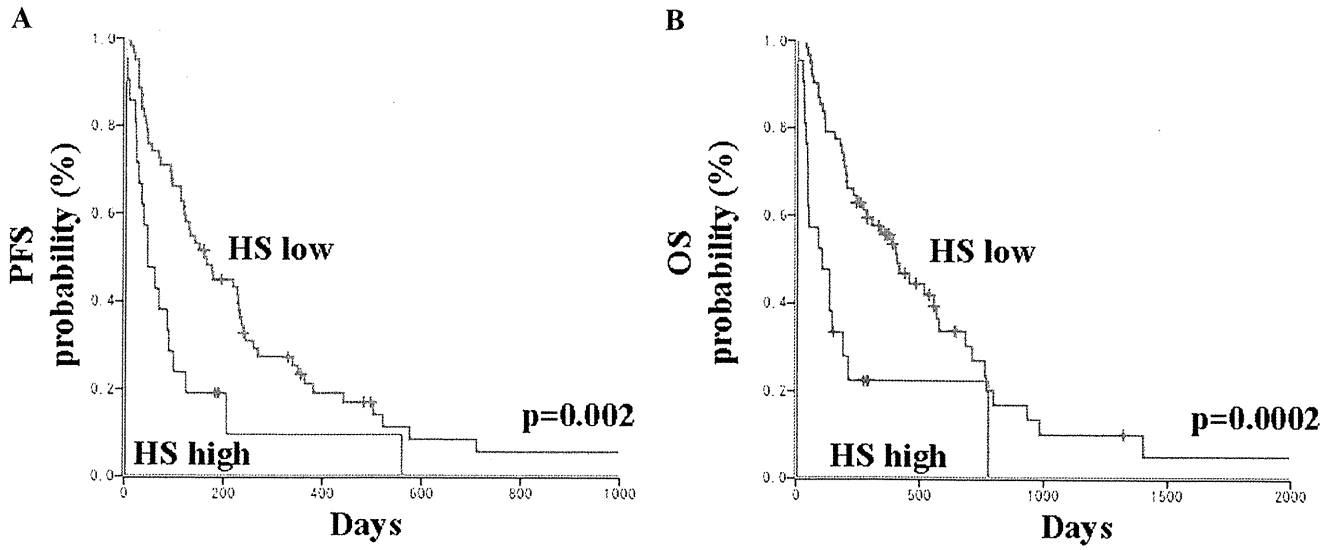

FIGURE 2. Kaplan-Meier curves for progression-free survival (PFS) and OS according to serum concentrations of heparan sulfate (HS). Optimal cutoff point (13.5 μg/ml) of serum HS was determined from a receiver operating characteristic (ROC) curve to discriminate progressive disease (PD) or without PD. Kaplan-Meier curves for PFS (*A*) and OS (*B*) are shown. HS high, patients with a serum HS concentration >13.5 μg/ml. HS low, serum HS concentration <13.5 μg/ml.

TABLE 3. Final Multivariate Model for Progression-Free Survival and Overall Survival

	PFS		OS	
	HR	*p*	HR	*p*
Sex				
(male vs. female)	1.82	0.0231	2.43	0.0031
PS				
(2–4 vs. 0–1)	1.09	0.7610	1.95	0.0235
HS[a]	3.98	0.0012	5.42	0.0003

Sex and PS were fixed in the model. Molecular markers were then selected using the backward selection procedure with a removal probability of 0.05. In all the steps, a Cox model stratified according to the EGFR status (wild type/mutant/unknown) was applied.

[a] Log-transformed values are used for HS.

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; EGFR, epidermal growth factor receptor; HS, serum concentration of heparan sulfate (μg/ml); PS, performance status.

and these high expression levels were predictors of a poor prognosis.[27,28] Compared with the normal form of syndecans-1, the shed form of syndecans-1 gains oncogenic functions leading to hyperinvasiveness and the increased tumor growth of myeloma tumors in vivo.[29] Thus, shed HSPGs remain highly biologically active and can regulate cell growth and metastasis in cancer.[30] In line with this observation, our findings that a high HS expression level was correlated with a poor clinical outcome may be associated with the expression of the shed/soluble form of HSPGs. Regarding the correlation between the HS expression levels in tumor and serum samples, we examined the HS expression levels in 10 independent pairs of serum and surgical samples. Representative results of the immunostaining for tumor HS expression are shown in Supplementary Figure 2 (http://links.lww.com/JTO/A110). An anti-HS antibody was used in this experiment with or without heparitinase I digestion. Heparitinase I digestion completely abolished the staining (left panel). Under such conditions, HS was strongly expressed on the membranes of lung cancer cells (lower right panel). Furthermore, HS expression in the tumor tissues was relatively weak in all five cases in the group with a low serum HS level (lower panel, Supplementary Figure 3, http://links.lww.com/JTO/A111), whereas strong HS expression in the tumor tissues was observed in three of the five cases in the group with a high serum HS level (upper panel, Supplementary Figure 3). These results suggested that the tumor and serum HS expression levels may be positively correlated.

The activation of EGFR signaling occurs as a result of mutations affecting the adenosine triphosphate-binding cleft of EGFR, and EGFR mutants exhibit constitutive tyrosine kinase activity independently of any ligand. We found that the serum HS concentration was significantly higher among patients with PD and was strongly associated with a poor PFS and OS in EGFR-TKIs-treated patients. No difference in the serum HS concentration was observed between the PR and SD groups, but a difference was seen between the PD and non-PD groups (Figure 1). Therefore, the serum HS concentration may be involved in drug resistance but not in sensitivity. Regarding resistance to EGFR-TKIs treatment, the amplification of met proto-oncogene (MET) causes gefitinib resistance by driving the v-erb-b2 avian erythroblastic leukemia oncogene homolog 3 (ERBB3)-dependent activation of phosphoinositide-3-kinase, and previous authors have proposed that MET amplification may promote drug resistance in other ERBB-driven cancers.[31] Yano et al.[32] showed that HGF-mediated MET activation is involved in gefitinib resistance in lung adenocarcinoma with EGFR-activating mutations. A recent study has clearly demonstrated that HGF accelerates the devel-

opment of MET amplification both in vitro and in vivo, mediating the EGFR kinase inhibitor resistance caused by either MET amplification or autocrine HGF production.[33] These studies indicate that the activation of HGF-MET signaling confers resistance to EGFR-TKIs. Our previous study also showed that a high concentration of serum HGF is a predictive biomarker for EGFR-TKIs treatment.[17] Therefore, the activation of HGF-MET signaling in lung cancer cells is considered to be a cause of drug resistance to EGFR-TKIs. In addition, combined with data on the serum HGF, VEGF, HB-EGF, and PDGF-BB levels from a previous study,[17] the correlation coefficient between the serum HS and the HGF, VEGF, HB-EGF, and PDGF-BB levels were 0.45, 0.46, −0.03, and −0.13, respectively. These results indicated that the expression pattern of the serum HS level was weakly similar to those of the HGF and VEGF levels but was not correlated with the HB-EGF or PDGF-BB levels.

In this study, the serum HS concentration was identified as another candidate biomarker for treatment resistance, and this finding may provide novel glycobiological insight into drug resistance to EGFR-TKIs. The results suggest that a high serum HS concentration may be related to the activation of non-EGFR signaling, such as HGF, FGF, and VEGF signaling in cancer cells. We plan to conduct a prospective study to validate the ability of the serum HS concentration to predict the response to EGFR-TKIs treatment.

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RESEARCH

MUC1 Expression in Pulmonary Metastatic Tumors: A Comparison of Primary Lung Cancer

Kyoichi Kaira · Takehiro Okumura · Kazuo Nakagawa · Yasuhisa Ohde · Toshiaki Takahashi · Haruyasu Murakami · Tateaki Naito · Masahiro Endo · Haruhiko Kondo · Takashi Nakajima · Nobuyuki Yamamoto

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Abstract MUC1 expression has been described as a predictor for tumor progression and worsening of prognosis in various human neoplasms. However, little is known about the role of MUC1 expression in pulmonary metastatic tumors. The aim of this study is to examine the clinicopathological significance of MUC1 expression in pulmonary metastatic tumors (PMT). One hundred forty-seven patients with PMT who underwent ^{18}F-FDG PET before metastasectomy were included in this study. Tumor sections were stained by immunohistochemistry for MUC1, glucose transporter 1 (Glut1), hypoxia-inducible-1α (HIF-1α) and vascular endothelial growth factor (VEGF). ^{18}F-FDG uptake and the expression of these biomarkers were correlated in primary lung cancer. MUC1 expression pattern was classified into high-grade polarized expression (HP), low-grade polarized expression (LP), or depolarized expression (DP) group. Of 147 patients, HP, LP and DP group were 9 (6%), 114 (78%) and 24 (16%), respectively. The expression of Glut1, HIF-1αand VEGF, and ^{18}F-FDG uptake were significantly higher in DP group than HP or LP groups. MUC1 expression with HP and DP pattern was significantly higher in primary lung cancer than in PMT, whereas, MUC1 expression with LP pattern yielded a significantly high positive rate in PMT. LP group was recognized in the majority of patients with pulmonary metastatic adenocarcinoma, especially colon cancer, whereas, HP group was significantly low in pulmonary metastatic adenocarcinoma as compared with primary adenocarcinoma. Polarized MUC1 has a different expression pattern between primary and metastatic tumors with adenocarcinoma, and depolarized MUC1 is closely associated with glucose metabolism and hypoxia.

Keywords MUC1 · Pulmonary metastatic tumor · NSCLC · ^{18}F-FDG PET · Glut1 · Hypoxia

K. Kaira (✉) · T. Takahashi · H. Murakami · T. Naito · N. Yamamoto
Division of Thoracic Oncology, Shizuoka Cancer Center, 1007 Shimonagakubo Nagaizumi-cho Sunto-gun, Shizuoka 411-8777, Japan
e-mail: kkaira1970@yahoo.co.jp

T. Okumura · K. Nakagawa · Y. Ohde · H. Kondo
Division of Thoracic Surgery, Shizuoka Cancer Center, 1007 Shimonagakubo Nagaizumi-cho Sunto-gun, Shizuoka 411-8777, Japan

M. Endo
Division of Diagnostic Radiology, Shizuoka Cancer Center, 1007 Shimonagakubo Nagaizumi-cho Sunto-gun, Shizuoka 411-8777, Japan

T. Nakajima
Division of Pathology, Shizuoka Cancer Center, 1007 Shimonagakubo Nagaizumi-cho Sunto-gun, Shizuoka 411-8777, Japan

Introduction

The impact of a strong expression of MUC1 mucin in various human neoplasms was repeatedly described as a predictor for tumor progression and worsening of prognosis [1–12]. Moreover, MUC1 has emerged as a target molecule in immunotherapy for various cancers [13]. As the mechanism of a target for cancer treatment, unmasked epitopes of MUC1 core protein expressed on tumor cells have been described to be able to elicit a strong antitumor immunity. But, the functional role of MUC1 expression is only partially elucidated.

Lung is one of the major metastatic sites of the neoplasm arising from other organs. Since it is sometimes difficult to

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differentiate metastatic pulmonary nodule from primary lung cancer, pulmonary metastastasectomy has become an integral part of diagnosis and treatment if the primary malignancies outside the thorax are controlled. As pulmonary metastatic tumor (PMT) is a heterogenous group of tumors, there is only limited data about the comparison of molecular biology between pulmonary metastatic tumors and primary lung cancer.

Recently, several reports have documented that the over-expression of MUC1 has a crucial role on the cancer progression and metastasis, leading to poor outcome, in patients with non-small cell lung cancer (NSCLC) [3, 4, 13–16]. However, the precise expression profiles of MUC1 have not been yet determinate in PMT. Little is known about how the expression of MUC1 differs between primary lung cancer and PMT.

The usefulness of 2-[^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) positron emission tomography (PET) can help predicting the therapeutic response and outcome in PMT patients [17]. The amount of ^{18}F-FDG uptake within tumor cells has been also documented to be determined by the presence of glucose metabolism [glucose transporter 1 (Glut1)], hypoxia [hypoxia-inducible factor-1α (HIF-1α)], and angiogenesis [vascular endothelial growth factor (VEGF)] [17, 18]. Recent experimental studies demonstrated that hypoxia enhances the expression of MUC1 through the direct regulation by HIF-1α in human cancer cell lines [19, 20]. Glut1 and VEGF could be regulated by HIF-1α-dependent way [17, 18], therefore, ^{18}F-FDG PET may be useful to evaluate whether hypoxia is associated with MUC1 expression in human neoplasm.

To elucidate the role of MUC1 expression in PMT, we conducted an immunohistochemical examination of MUC1 in patients with PMT, which was compared with primary lung cancer. In addition, MUC1 expression was correlated with Glut1, HIF-1α, VEGF, and ^{18}F-FDG uptake within tumor cells.

Material and Methods

Patients

We analyzed 170 consecutive patients who underwent ^{18}F-FDG PET and lung resection for pulmonary metastasis from extrathoracic malignancies at Shizuoka Cancer Center between April 2003 and May 2009. The patients who underwent PET study prior to pulmonary metastasectomy were included, and the patients with other malignancies and those who received induction chemotherapy or radiation before pulmonary metastasectomy were excluded from this study. Six patients who received induction chemotherapy or radiation therapy were excluded. Specimens of seven patients were not available. Ten patients were excluded from analysis because they did not have ^{18}F-FDG PET within 4 weeks before their pulmonary resection was performed. Thus, a total of 147 patients who underwent pulmonary metastasectomy were analyzed in the study. All patients were imaged on ^{18}F-FDG PET.

As a test group of pulmonary malignancy, we evaluated MUC1 expression and the biomarkers including ^{18}F-FDG PET in patients with NSCLC, as compared with PMT. One hundred thirty-three NSCLC patients were consecutively assigned in the study between October 2002 and May 2004, and ^{18}F-FDG PET was performed as part of the preoperative workup. These patients underwent surgical management, and the primary lesions were surgically resected. Finally, a total of 126 patients (81 men, 45 women) were eligible in the study. These 126 patients have no pulmonary metastatic tumors due to primary malignancies outside the thorax. Histologically, 82 patients had AC, 36 had SQC, and 8 had other histology. Of the total patients, 63, 25 and 38 had stage I, II and III tumors, respectively. The study protocol was approved by the institutional review board.

Immunohistochemical Staining

Immunohistochemical staining was performed according to the procedure described in the previous reports [3, 17, 18]. The following antibodies were used: a rabbit monoclonal antibody against MUC1 (Ma 552; Novocastra; 1:100 dilution); a rabbit polyclonal antibody against GLUT1 (AB15309, Abcam, Tokyo, Japan, 1:200 dilution); a mouse monoclonal antibody against HIF-1α (NB100-123, Novus Biologicals, Inc., Littleton, 1:50 dilution); a monoclonal antibody against VEGF (Immuno-Biological Laboratories Co.,Ltd., Japan, 1:300 dilution).

According to previous report [3], immunohistochemical analysis of MUC1 expression was evaluated. Firstly, staining density of MUC1 expression was classified into positive or negative, and if positive, each tumor cell was further classified according to the expression pattern into polarized or depolarized expression. According to the percentage of tumor cells showing polarized MUC1 expression and that with depolarized MUC1 expression, MUC1 expression was classified into the high-grade polarized (HP), the low-grade polarized (LP), or the depolarized (DP) group. The classification of MUC1 expression status is as follows: (i) HP when positive percentage of tumor cells with polarized MUC1 expression is more than 50% and positive percentage of tumor cells with depolarized MUC1 expression is less than 10%, (ii) LP when positive percentage of tumor cells with polarized MUC1 expression is less than 50% and positive percentage of tumor cells with depolarized MUC1 expression is less than 10%, (iii) DP when positive percentage of tumor cells with depolarized MUC1 expression is more than 10% regardless of positive percentage of with polarized MUC1 expression. According to

the definition, the patient with tumor showing no MUC1 expression was classified into the LP group.

The expression of Glut1 was considered positive if distinct membrane staining was present. Five fields (X400) were analyzed to determine the frequency of the HIF-1α stained nuclei. For Glut1 and HIF-1α, a semi-quantitative scoring method was used: 1=<10%, 2=10–25%, 3=25–50%, 4=51–75% and 5=>75% of cells positive. The tumors in which stained tumor cells made up more than 25% of the tumor were graded as positive. The expression of VEGF was quantitatively assessed according to the percentage of immunoreactive cells in the total of 1,000 neoplastic cells.

^{18}F-FDG PET Imaging

Patients fasted for at least 4 h before ^{18}F-FDG PET examination. Patients received an intravenous injection of 200–250 MBq of ^{8}F-FDG and then rested for approximately 1 h before undergoing imaging [17, 18]. Image acquisition was performed using an Advance NXi PET scanner and Discovery PET-CT scanner (GE Medical Systems, Milwaukee, WI, USA). Two-dimensional emission scanning was performed from the groin to the top of the skull. PET/CT image was independently reviewed by two experienced physicians. Acquired data were reconstructed by iterative ordered subset expectation maximization. To evaluate ^{18}F-FDG accumulation, the tumor was first examined visually, and then the peak standardized uptake value (SUV) of the entire tumor was determined. SUV_{max} was defined as the peak SUV value on one pixel with the highest counts within the region of interest (ROI). The ROI, measuring 3 cm in diameter, was set at the mediastinum at the level of the aortic arch and the mean SUV of the mediastinum was calculated. Finally, the T/M ratio, which is the ratio of the peak SUV of the tumor to the mean SUV of the mediastinum, was determined for each patient.

Statistical Analysis

Probability values of <0.05 indicated a statistically significant difference. Fisher's exact test was used to examine the association of two categorical variables. Correlation of different variables was analyzed using the nonparametric Spearman's rank test. Statistical analysis was performed using JMP 8 (SAS, Institute Inc., Cary, NC, USA) for Windows.

Results

Patient Characteristics

The median age of the patients was 64 years (range, 16–82 years). Eighty-one patients were men and 66 were women. The tumor size of resected metastatic tumors ranged from 5 to 68 mm (median, 14 mm). Eastern Cooperative Oncology Group (ECOG) performance status (PS) was 0–1 in all patients. Seventy-five (51%) of 147 patients were smokers. Fifty-seven patients received adjuvant chemotherapy after pulmonary metastasectomy. The organ types of the primary site were as follows: 80 colon cancers, 7 breast cancers, 14 head and neck cancers, 12 soft-tissue sarcomas, 19 genital cancers, 12 gastrointestinal cancers and 3 other cancers. Forty (50%) of 80 patients with colon cancers have a primary site of rectum. Of 12 gastrointestinal cancers, 5 patients have esophageal cancer with SQC and 7 patients gastric cancer with AC. Of 12 sarcomas, 7 patients have osteosarcoma, 3 patients synovial sarcoma and 2 patients malignant fibrous histiocytoma. In NSCLC group, the median size of the resected lesions was 23 mm (range, 6 to 100 mm).

Immunohistochemical Analysis and ^{18}F-FDG PET Findings

Each protein revealed a profile pattern of the unique expression. The immunohistochemical staining was evaluated for the surgically resected 147 pulmonary metastatic lesions. Figure 1 represents the immunohistochemical staining of MUC1 expression. Of all 147 patients, HP, LP and DP group were 9 (6%), 114 (78%) and 24 (16%), respectively. The frequency of LP group was significantly higher than that of HP and LP groups (p<0.0001). Glut1 was detected in tumor cells and localized predominantly on their plasma membrane. A positive rate of Glut1 expression was recognized in 70%. A positive expression of HIF-1α was predominantly expressed in the cytoplasm with some nuclear staining, and was recognized in 70%. The staining pattern of VEGF was uniformly localized in the cytoplasm and/or membrane. The median rate of VEGF positivity was 22.0% (range, 2–76%), and the value of 22% was chosen as a cutoff point. High expression was recognized in 50%.

The mean values (mean and standard deviation) of T/M ratio in PMT and NSCLC were 3.25±0.22 (range, 0.95 to 9.43) and 5.96±0.38 (range, 0.8 to 24.0), respectively. The T/M ratio of PMT was significantly lower than that of NSCLC (p<0.0001). Of patients with NSCLC, The mean values of T/M ratio in AC and SQC were 4.93±0.51 (range, 0.8 to 21.5) and 7.32±0.73 (range, 2.4 to 24.0), respectively, demonstrating statistically significant difference. The T/M ratio of PMT was significantly lower than that of primary lung SQC, but was not than primary lung AC. The median value of T/M ratio in PMT was 3.0, and a median value of 3.0 was used as the cutoff T/M ratio in following analyses. The T/M ratio of more than 3.0 was defined as high expression.

Figure 2 shows the expression of these biomarkers and T/M ratio of ^{18}F-FDG uptake according to MUC1 expression. In PMT patients, the mean scoring of Glut1 and HIF-1α,

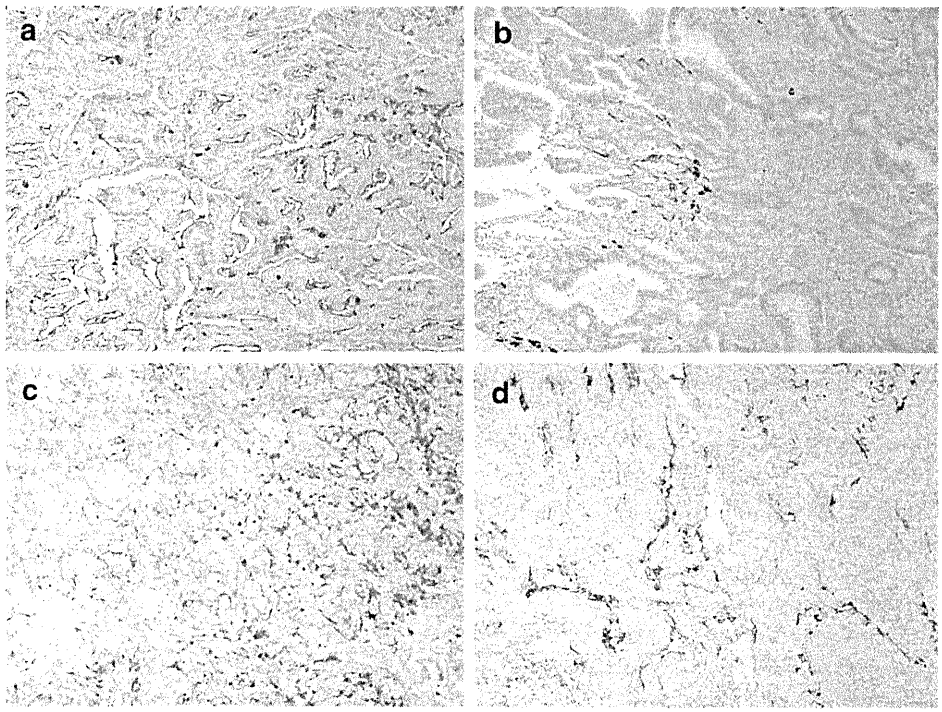

Fig. 1 Immunohistochemical staining of MUC1 expression in pulmonary metastatic tumors: **a** High-grade polarized expression (HP) pattern of MUC1 expression in breast cancer. **b** Low-grade polarized expression (LP) pattern of MUC1 expression in colon cancer. **c** Depolarized expression (DP) pattern of MUC1 in renal cell carcinoma. Immunohistochemical staining of MUC1 expression in primary lung cancer: **d** High-grade polarized expression (HP) pattern of MUC1 expression in pulmonary adenocarcinoma

VEGF positivity, and T/M ratio of ^{18}F-FDG uptake were significantly higher in DP group than HP or LP groups, demonstrating no significant difference between HP and LP groups (Fig. 2a). In patients with primary lung AC, the mean scoring of Glut1 and HIF-1α, VEGF positivity, and T/M ratio of ^{18}F-FDG uptake were significantly higher in LP group than DP group (Fig. 2b). No statistically significant difference in the uptake of ^{18}F-FDG and the meaning scoring of Glut1 and VEGF was observed between HP and LP groups, but uptake of ^{18}F-FDG, the mean scoring of Glut1 and VEGF positivity yielded a statistically significant difference between HP and DP groups. In patients with primary lung SQC, no statistically significant difference in these biomarkers was recognized between HP and LP, between LP and HP, and between HP and DP (Fig. 2c)

Relationship Between MUC1 Expression and Different Variables

The demographic result of the patients according to MUC1 expression is listed in Table 1. The frequency of young age, multiple metastases, large tumor size and a positive Glut1 expression was significantly higher in DP group than in HP group. A statistically significant difference in the age was observed between HP and LP group. The frequency of positive Glut1, HIF-1α and VEGF expression was significantly higher in DP group than in LP group.

We analyzed the expression of MUC1 according to histological types in PMT (Fig. 3a). One hundred and one patients had AC, 15 patients had SQC and 20 patients had sarcoma. In HP group, no significant difference in the positive rate of MUC1 expression was observed among AC, SQC and sarcoma patients. The positive rate of MUC1 expression with LP pattern was significantly higher in AC patients than in SQC patients. But, the positive rate with DP pattern was significantly lower in AC patients than in SQC patients.

According to the organ of the primary sites, the positive rate of MUC1 expression was examined (Fig. 3b). In colon cancer and soft-tissue sarcoma, the positive rate of MUC1 expression with LP pattern was significantly higher than that with HP or DP pattern. In head and neck cancer, MUC1 expression was significantly higher in LP pattern than in HP pattern. In genital cancer, MUC1 expression was significantly higher in DP pattern than HP pattern.

Next, we compared the expression of MUC1 between NSCLC and PMT (Fig. 3). In the analysis of total patients, the MUC1 expression with HP and DP pattern was significantly higher in NSCLC than in PMT, whereas, the MUC1 expression with LP pattern in PMT yielded a significantly high positive rate as compared with NSCLC (Fig. 3c). In AC patients, MUC1 expression with LP pattern was significantly higher in PMT than in NSCLC, whereas, the MUC1 expression with HP pattern in NSCLC yielded a significantly high positive rate as compared with PMT (Fig. 3d). In SQC patients, no significant difference in the positive rate of MUC1 expression was observed between NSCLC and PMT (Fig. 3e).

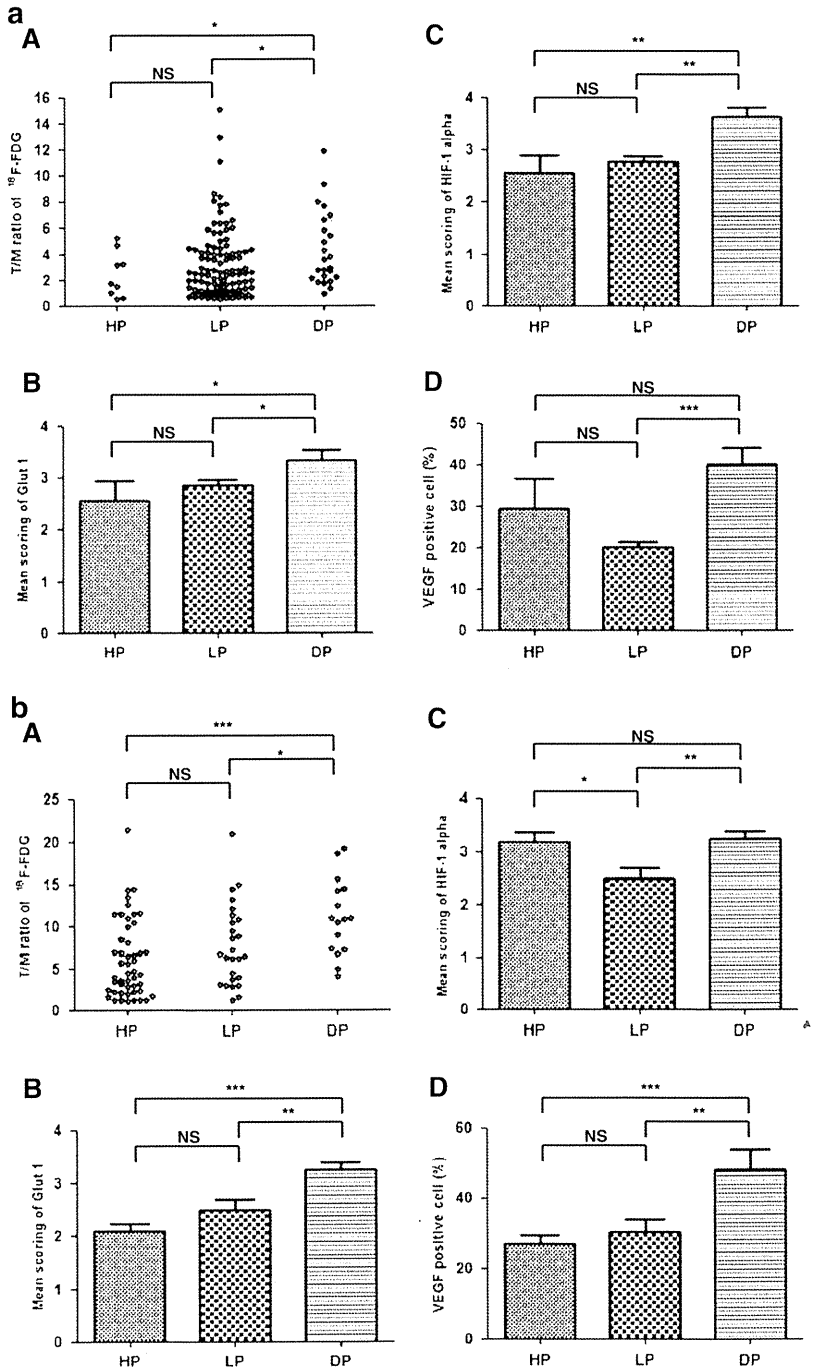

Fig. 2 Comparison of ^{18}F-FDG uptake and angiogenic markers according to MUC1 expression: HP, high-grade polarized expression; LP, low-grade polarized expression; DP, depolarized expression. **a** T/M ratio of ^{18}F-FDG uptake, the mean scoring of Glut1 and HIF-1α, and VEGF positivity of patients with pulmonary metastatic tumors according to MUC1 expression pattern. **b** T/M ratio of ^{18}F-FDG uptake, the mean scoring of Glut1 and HIF-1α, and VEGF positivity of patients with primary lung AC according to MUC1 expression pattern. *P* values indicate significance and were calculated using Fisher's exact test. **c** T/M ratio of ^{18}F-FDG uptake, the mean scoring of Glut1 and HIF-1α, and VEGF positivity of patients with primary lung SQC according to MUC1 expression pattern. *P* values indicate significance and were calculated using Fisher's exact test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. NS, not significant

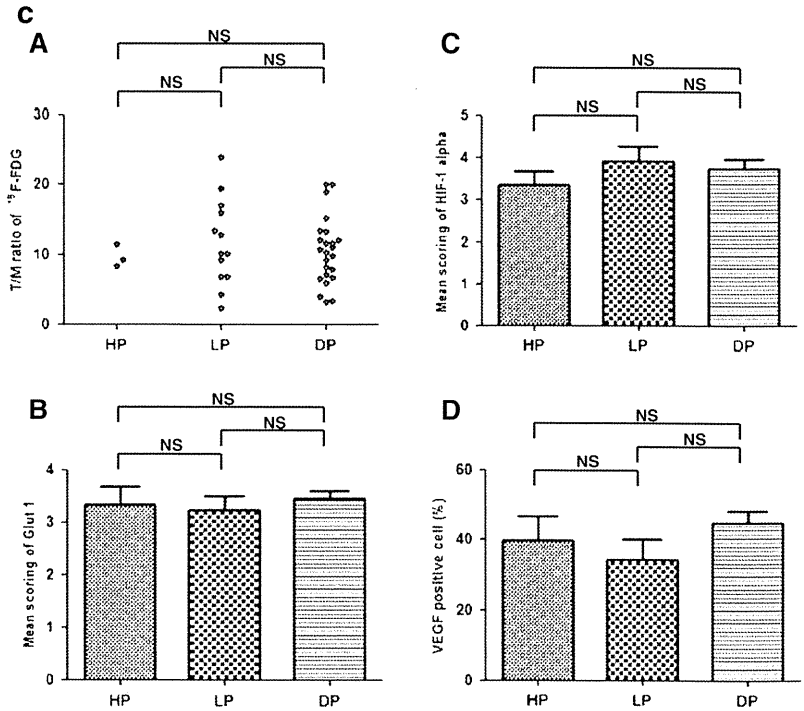

Fig. 2 (continued)

Discussion

This is a clinicopathological study to investigate the expression of MUC1 expression in patients with PMT as compared with NSCLC. MUC1 expression with LP pattern was observed in almost patients with PMT, especially colon cancer and soft-tissue sarcoma. In AC patients, the frequency of LP pattern was significantly higher in PMT tumors than in NSCLC, and MUC1 expression with HP pattern in NSCLC yielded a significantly high positive rate as compared with PMT. A high ^{18}F-FDG uptake in PMT was observed in DP pattern as compared to HP pattern, and the expression of Glut1 and HIF-1α were significantly higher in DP pattern

Table 1 Patient's demographics according to MUC1 expression

Different variables		Total (n=147)	HP (n=9)	LP (n=114)	DP (n=24)	p-value		
						HP/LP	HP/DP	LP/DP
Age	(≤ 65 / > 65 yr)	77 / 70	1 / 8	62 / 52	14 / 10	0.015	0.021	0.822
Gender	(Male / Female)	78 / 69	2 / 7	64 / 50	12 / 12	0.079	0.240	0.654
Smoking	(Yes / No)	76 / 71	2 / 7	63 / 51	11 / 13	0.082	0.263	0.500
PS	(0 / 1)	124 / 23	7 / 2	97 / 17	20 / 4	0.628	1.000	0.762
Tumor size	(≤ 15 / > 15 mm)	63 / 84	7 / 2	48 / 66	8 / 16	0.076	0.046	0.497
No. of meta	(Single / Multiple)	121 / 26	5 / 4	94 / 20	22 / 2	0.071	0.034	0.365
Adjuvant CTx	(Yes / No)	63 / 84	3 / 6	55 / 59	5 / 19	0.497	0.651	0.022
T/M ratio	(High / Low)	58 / 89	4 / 5	42 / 72	12 / 12	0.726	1.000	0.255
Glut 1	(Positive / Negative)	106 / 41	5 / 4	79 / 35	22 / 2	0.462	0.034	0.023
HIF-1α	(Positive / Negative)	103 / 44	6 / 3	75 / 39	22 / 2	1.000	0.110	0.012
VEGF	(Positive / Negative)	71 / 76	5 / 4	45 / 69	21 / 3	0.483	0.068	< 0.01

HP high-grade polarized expression; *LP* low-grade polarized expression; *DP* depolarized expression; *PS* performance status; *No. of meta* Number of resected metastases; *Adjuvant CTx* adjuvant chemotherapy; *Glut1* glucose transporter 1; *HIF-1α* hypoxia inducible factor-1 alpha; *VEGF* vascular endothelial growth factor; *HP/LP* statistical comparison of HP and LP; *HP/DP* statistical comparison of HP and DP; *DP/LP* statistical comparison of DP and LP

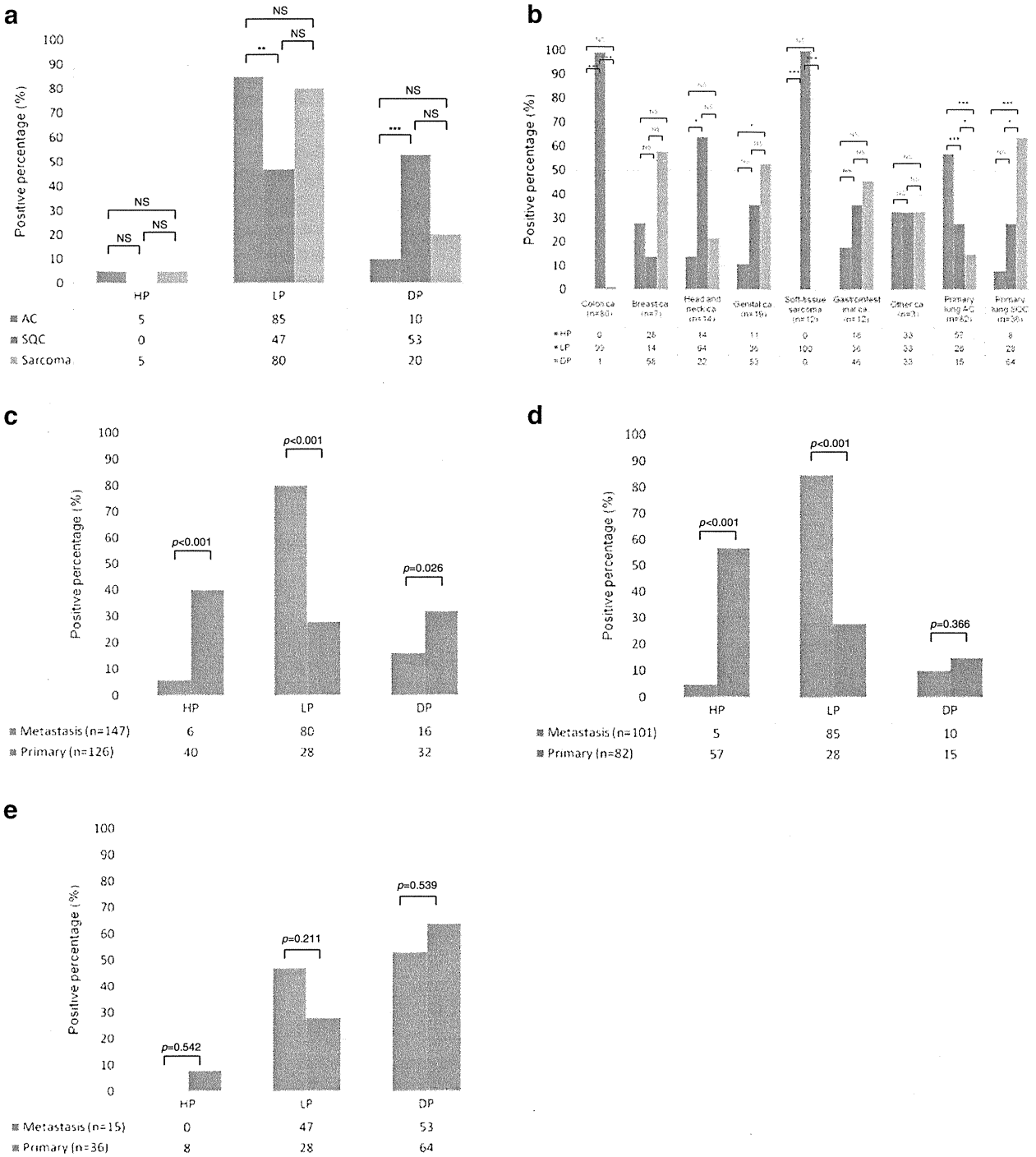

Fig. 3 Comparison of MUC expression according to primary sites and histological types: HP, high-grade polarized expression; LP, low-grade polarized expression; DP, depolarized expression; AC, adenocarcinoma; SQC, squamous cell carcinoma. **a** MUC1 expression according to histological types in pulmonary metastatic tumors. **b** Positive rate of MUC1 expression according to the organ of the primary sites. Comparison of MUC1 expression between primary lung cancer and pulmonary metastatic tumors in total patients (**c**), AC patients (**d**) and SQC patients (**e**). *P* values indicate significance and were calculated using Fisher's exact test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. NS, not significant

than in HP pattern. This was corresponding to the results of pulmonary lung AC.

MUC1 is a transmembrane mucin consisting of a heavily *O*-glycosylated excellilar domain, a transmembrane domain

and a cytoplasmic tail of 72 amino acids [1]. Recently, several reports have documented that MUC1 expression is correlated with tumor differentiation and postoperative survival in patients with NSCLC [4, 14–16], and Nagai et al has described that depolarized MUC1 expression was a significant and independent prognostic factor to predict poor postoperative prognosis in patients with pulmonary adenocarcinoma and LP or DP expression was mostly observed in moderately to poorly differentiated adenocarcinoma patients [3]. Nagai et al conducted a more detailed MUC1 status classification (HP, LP and DP) for the immunohistochemical evaluation of MUC1 expression in pulmonary tumors [3]. In previous literatures, the immunohistochemical analyses of MUC1 expression were different among the primary tumors and the studies, and the methods used in the studies also have a different technique [4–12]. To analyze the MUC1 expression of pulmonary tumors, therefore, we selected the expression analysis of MUC1 according to Nagai's study [3]. In this study, we could directly compare the expression of MUC1 between NSCLC and PMT.

In our study, low-grade polarized MUC1 expression was observed in the majority of patients with PMT, especially adenocarcinoma such as colon cancer or soft-tissue sarcoma. On the other hand, the frequency of high-grade polarized MUC1 expression was significantly low in pulmonary metastatic adenocarcinoma as compared with primary adenocarcinoma. Only small number of patients with PMT showed the expression pattern of high-grade polarized MUC1, and depolarized MUC1 expression was mainly observed in patients with SQC or genital cancers. In patients with AC as pulmonary nodules, the primary sites are sometimes difficult to differentiate between primary lung cancer and extrathoracic tumor. However, our results suggest that polarized MUC1 (HP or LP pattern) has a markedly different expression pattern between primary and metastatic pulmonary tumors with a histological type of AC. In patients with SQC as pulmonary nodules, whereas, it is difficult to differentiate NSCLC from PMT, because the expression profile of MUC1 was similar among these groups. In addition, ^{18}F-FDG uptake within tumor cells tended to increase from HP, LP to DP pattern, and the expression of Glut1 and HIF-1α was also significantly higher in DP pattern than in HP or LP pattern. Hypoxia has been documented to enhance MUC1 expression in human cancer cell lines, and the present study suggested that hypoxia and glucose metabolism were closely associated with the expression of depolarized MUC1 as compared with that of polarized MUC1. In clinical practice, ^{18}F-FDG PET may be effective for differentiating between polarized MUC1 and depolarized MUC1 expression tumors. However, ^{18}F-FDG PET was not useful for differentiating between HP and LP pattern of MUC1 expression in PMT patients.

MUC1 core protein may be a useful target molecule for immunotherapy in breast cancer, lung cancer and other malignancies expressing MUC1 [21, 22]. MUC1-targeted immunotherapy may be appropriate for such patients as postoperative adjuvant therapy. However, it remains unclear whether MUC1 expression is associated with postoperative outcome in patients with PMT. If not investigate the relationship between MUC1 expression and prognosis, it seems to be difficult to speculate the possibility of a MUC1-targeted immunotherapy after pulmonary metastasectomy in patients with PMT.

In conclusion, polarized MUC1 (HP or LP pattern) had a markedly different expression pattern between primary and metastatic pulmonary tumors with a histology of AC, and depolarized MUC1 was closely associated with glucose metabolism and hypoxia. In addition, ^{18}F-FDG PET may be effective for differentiating between polarized MUC1 and depolarized MUC1 expression tumors. Further study is warranted for investigating the possibility of a MUC1-targeted immunotherapy as a postoperative adjuvant chemotherapy after pulmonary metastasectomy.

Conflicts of Interest Statement We, all authors, have no financial or personal relationships with other people or organizations that could inappropriately influence our work.

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Short Communication

Serum Brain-derived Neurotrophic Factor and Antidepressant-naive Major Depression After Lung Cancer Diagnosis

Makoto Kobayakawa[1,2], Masatoshi Inagaki[3,4], Maiko Fujimori[1], Kei Hamazaki[5], Tomohito Hamazaki[5], Tatsuo Akechi[1,6], Shoichiro Tsugane[7], Yutaka Nishiwaki[8], Koichi Goto[8], Kenji Hashimoto[9], Shigeto Yamawaki[2] and Yosuke Uchitomi[1,10,*]

[1]Psycho-Oncology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa-City, Chiba, [2]Psychiatry Division, Graduate School of Biomedical Science, Hiroshima University, Hiroshima-City, Hiroshima, [3]Center for Suicide Prevention, National Institute of Mental Health, National Institute of Neurology and Psychiatry, [4]Department of Psychogeriatrics, National Institute of Mental Health, National Institute of Neurology and Psychiatry, Kodaira-City, Tokyo, [5]Division of Clinical Application, Department of Clinical Sciences, Institute of Natural Medicine, University of Toyama, Toyama-City, Toyama, [6]Department of Psychiatry and Cognitive-Behavioral Medicine, Nagoya City University Graduate School of Medical Science, Nagoya-City, Aichi, [7]Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Chuo-ku, Tokyo, [8]Thoracic Oncology Division, National Cancer Center Hospital East, Kashiwa-City, [9]Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, Chiba and [10]Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama-City, Okayama, Japan

*For reprints and all correspondence: Yosuke Uchitomi, Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science, 2-5-1 Shikata-cho, Kita-ku, Okayama-City, Okayama 700-8556, Japan. E-mail: uchitomi@md.okayama-u.ac.jp

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Previous studies have reported the existence of an association between brain-derived neurotrophic factor and major depression. However, the possible role of brain-derived neurotrophic factor in the pathophysiology of major depression after cancer diagnosis has not yet been investigated. Subjects were collected using the Lung Cancer Database project. Using the cut-off scores on the depression subscale of the Hospital Anxiety and Depression Scale (HADS-D), 81 subjects with depression (HADS-D $>$ 10) and 81 subjects without depression (HADS-D $<$ 5) were selected. The two groups were matched for age, sex, clinical stage and performance status. The serum brain-derived neurotrophic factor levels were measured using an enzyme-linked immunosorbent assay method. The serum brain-derived neurotrophic factor levels were not statistically different between the subjects in the depression group [29.1 (13.6) ng/ml; mean (SD)] and the non-depression group [31.4 (10.6) ng/ml] ($P = 0.22$). In a stratified analysis by gender, however, the mean serum brain-derived neurotrophic factor level in the depression group tended to be lower than that in the non-depression group among women ($n = 24$ pairs, $P = 0.06$). Major depression after cancer diagnosis is not associated with serum brain-derived neurotrophic factor levels.

Key words: major depression – BDNF – lung cancer – cancer diagnosis – stressful event

INTRODUCTION

Cancer is a common and worldwide fatal disease. Learning about the diagnosis of cancer is an extremely stressful life event, and major depression is common among patients with cancer (1). Stressful events are usually considered as strong risk factors for major depression (2). Therefore, the high

prevalence of major depression among cancer patients may be attributable to cancer-specific stressful events (3). However, the pathway by which stressful events lead to major depression among cancer patients has not yet been elucidated.

Recently, brain-derived neurotrophic factor (BDNF) has been recognized as an important factor in the pathophysiology of stress-related mental disorders, particularly major depression (4). In animal studies, the relationships between the stress and decreased expression of BDNF mRNA in the hippocampus and neocortex of rats (5,6), and increased synthesis of BDNF induced by interventions like depression treatments (7,8) were suggested. Patients with major depression had lower levels of serum BDNF than healthy controls (9–11), and the levels of serum BDNF changed to be normal after treatment for depression (9,11). However, with no such studies in the oncologic setting, we preliminarily planed to examine the difference in serum BDNF levels between subjects with and without antidepressant-naive major depression after being diagnosed as having lung cancer, which is a stressful life event and was not considered in the previous human studies (9–11). We hypothesized that the serum BDNF levels in the subjects developing major depression after being diagnosed as having lung cancer would be lower than in those without depression. We secondarily performed a stratified analysis by gender, because a previous study showed significantly low serum BDNF levels in depressive women, but not in depressive men (11).

PATIENTS AND METHODS

Study Design and Subjects

The present study used secondary samples from our previous study (12) on the Lung Cancer Database project (13). The project was a prospective cohort study to investigate the pathogenesis of and the development of new therapy for lung cancer. The project and the present study were approved by the Institutional Review Board and the Ethics Committee of the National Cancer Center, Japan. All participants provided their written informed consent prior to enrollment.

The details of the inclusion and exclusion criteria of the present study were described in our previous report (12). In concise, patients newly diagnosed as having primary lung cancer were included, and patients with cognitive impairment, past or current histories of mental disorders, and brain neoplasms or brain metastasis were excluded. To remove the influence of severe physical status, patients with a performance status (PS) of 2 or higher were also excluded (PS was defined by Eastern Cooperative Oncology Group).

Assessment of Depression

Self-reported questionnaires, including the Hospital Anxiety and Depression Scale (HADS) (14), were completed during the waiting period prior to admission. The HADS consists of seven-item anxiety and seven-item depression subscales and is used to assess anxiety and depressive symptoms during the preceding week. The Japanese version of the depression subscale of the HADS (HADS-D) has two cut-off points that yield a good sensitivity and specificity for depression screening (10 out of 11; 82.4 and 95.1%, major depression only, 4 out of 5; 91.5 and 58.0%, adjustment disorder and major depression, respectively) (15). In this study, 'depression' was defined based on HADS-D scores without usual procedure such as the Structured Clinical Interview for DSM-IV.

Selection of Depression and Non-depression Groups

Subjects were selected according to the method used in our previous study (12), as follows: (i) all eligible subjects were classified into three groups according to the two cut-off points (10 out of 11 and 4 out of 5) for HADS-D; (ii) the number of subjects in the high-score group (>10) was used as the number of cases with major depression; (iii) the same number of controls in the low-score group (<5) was selected from the eligible subjects so that the two groups were matched for age, sex, PS (0 or 1) and clinical stage as assessed by the TNM classification (Ia–IIIa or IIIb–IV). To compare major depression with non-depression, the cases with high HADS-D scores (>10) were enrolled in the 'depression group', and the cases with low scores (<5) were included in the 'non-depression group'.

Measurement of Serum BDNF

Following an overnight fast, blood samples were collected by registered nurses in the morning (7–9 AM), a few days after admission. After storing the samples for about 2 h at 4°C, the serum was separated by centrifugation (1870*g*, 10 min) and stored at −80°C until further assay. The samples were thawed to 4°C and the serum BDNF levels were measured using an enzyme-linked immunosorbent assay kit (Promega, Madison, WI, USA) (9). The absorbance of samples at 450 nm was measured using an Emax automated microplate reader (Molecular Device, Tokyo, Japan).

Assessment of Demographical and Medical Backgrounds

Information regarding clinical, demographic and social factors were collected from the database and the patients' medical charts (13). These data consisted of sex, age, clinical staging as assessed by the TNM classification, PS, pathologic type of the lung cancer, educational level (longer/not longer than 9 years), smoking status, alcohol consumption status, presence/absence of breathlessness and pain, number of platelets and body mass index.