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Clinical significance of the levels of platelet-derived microparticles, PAI-1 and HMGB1 as prognostic biomarkers for patients with non-small-cell lung cancer

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ABSTRACT

We measured various biomarkers in non-small-cell lung cancer (NSCLC) patients in an effort to discover novel prognostic indicators. We identified three such markers, namely platelet-derived microparticles (PDMP), plasminogen activator inhibitor (PAI)-1 and high-mobility group box-1 (HMGB1). Using multivariate analysis, we assessed 147 NSCLC patients and 35 control patients and found that HMGB1 and PAI-1 levels were significantly correlated with those of PDMP. We also analyzed the contribution of the newly designed risk factor (NDRF) to overall survival (OS) or disease-free survival (DFS). NDRF classification was determined based on levels of PDMP, HMGB1 and PAI-1. To determine the individual prognostic power of PDMP, HMGB1 and PAI-1, we evaluated associations between their levels and patients outcome by Kaplan–Meier survival analysis in the derivation cohort. NDRF3 status (patients who had high levels of all of PDMP, HMGB1 and PAI-1) was significantly correlated with a poor prognosis ($p < 0.05$ for both OS and DFS). Our findings suggest that abnormal levels of PDMP, HMGB1 and PAI-1 are related to each other in NSCLC. Moreover, the vascular complications associated with these three markers may contribute to a poor prognosis for NSCLC patients.

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KEYWORDS: non-small cell lung cancer, PAI-1, prognosis, platelet-derived microparticle, HMGB1

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. Eighty-five percent of all lung cancers are non-small-cell lung cancer (NSCLC), and approximately two-thirds of NSCLCs are at an advanced stage at diagnosis [2]. The current prognosis for patients with advanced NSCLC remains poor despite enhanced treatment strategies [3, 4]. Several biomarkers have emerged as potential prognostic and predictive markers for NSCLC, including epidermal growth factor receptor (EGFR) [5], high-mobility group box-1 (HMGB1) [6], mean platelet volume (MPV) [7, 8], neutrophil or platelet/lymphocyte rates [9, 10] and microparticle (MP) levels [11, 12]. Additionally, the utility of an inflammation-related index has been reported [13]. However, the reliability and usefulness of these markers are controversial at present.

Many individuals with cancer are also in a hypercoagulable state, and the elevated risk of thrombosis conferred by hypercoagulativity increases patient morbidity and mortality [14]. Cancer patients frequently develop venous thromboembolism (VTE), and various potential predictive biomarkers have been evaluated for their association with VTE in cancer progression [15-18]. For example, analysis of blood cells can effectively predict

the risk of VTE development [16]. Additionally, measurement of D-dimer, prothrombin fragment 1+2 and soluble P-selectin levels can accurately predict VTE risk [18]. Furthermore, MP levels are also an accurate marker of VTE risk [19-21].

Here, we evaluated the utility of multiple potential prognostic biomarkers in NSCLC patients. We identified platelet-derived MP (PDMP), plasminogen activator inhibitor (PAI)-1 and HMGB1 as useful prognostic indicators in NSCLC patients. To our knowledge, this is the first report detailing the relationship and clinical significance of these biomarkers for NSCLC prognosis.

MATERIALS AND METHODS

Subjects

NSCLC patients and healthy volunteers were recruited from Kansai Medical Hirakata Hospital and Kansai Medical University (Osaka, Japan) from September 2011 to July 2014. NSCLC patients were grouped according to the guidelines for tumor-node-metastasis stage I-IV, based on primary tumor size, lymph node involvement, and distance between the metastasis and primary tumor [22]. This study was conducted in accordance with the Declaration of Helsinki, and was performed with approval from the Institutional Review Board. Written informed consent was obtained from all participants.

Measurement of Interleukin (IL)-6, Monocyte-chemotactic Protein (MCP)-1, Regulated on Activation Normally T-cell Expressed and Secreted (RANTES), Vascular Cell Adhesion Molecule (VCAM)-1, E-selectin, High Mobility Group Box 1 (HMGB1), Angiopoietin (Ang)-2, Vascular Endothelial Growth Factor (VEGF), and PAI-1

Patient blood samples were collected in plain or sodium citrate-containing tubes and left at room temperature for a minimum of 1 h. Serum and citrated plasma were isolated by 20 min centrifugation at 1,000 × g at 4 °C. Serum was divided into aliquots and frozen at -30 °C until use. Recombinant products and standard solutions provided with commercial kits served as positive controls. Human IL-6, MCP-1, RANTES, sVCAM-1, sE-selectin and PAI-1 ELISA kits were purchased from Invitrogen International, Inc. (Camarillo, CA, USA). HMGB1 was measured using the HMGB1 ELISA Kit II (Shino-test Corp., Kanagawa, Japan). Serum levels of cytokines and soluble factors were measured according to the manufacturer's instructions. Normal ranges were as follows: IL-6: 0.2-4.5 pg/mL, MCP-1: 170-570 pg/mL, RANTES: 23.9-58.5 ng/mL, sVCAM-1: 395-714 ng/mL, sE-selectin: 23.0-79.2 ng/mL, HMGB1: 1.2-4.8 ng/mL, Ang-2: 500-2000 pg/mL, VEGF: 40-500 pg/mL, and PAI-1: 1.1-10.5 ng/mL.

Assessment of platelet-derived microparticle (PDMP)

Blood samples were collected with a 21-gauge needle from a peripheral vein into vacutainers containing EDTA-ACD (NIPRO Co. Ltd., Japan) to minimize platelet activation. The samples were handled as described in the manufacturer's protocol. In brief, the samples were gently mixed by inverting the tube once or twice, stored at room temperature for 2-3 h and centrifuged at 8,000 × g for 5 min at room temperature. Storage of samples at room temperature for 2-3 h did not affect the PDMP level. Immediately after centrifugation, 200 µL of upper-layer supernatants from 2-mL samples were collected to avoid contamination of the platelets [23, 24] and the samples were stored at -40 °C until analysis. PDMP levels were measured in duplicate using an ELISA kit by monoclonal antibodies against glycoprotein CD42a and 42b (JIMRO Co. Ltd., Japan) [23-27]. The range of normal PDMP values was 3-8 U/mL.

Statistical analysis

Results are shown as the mean ± standard errors. Statistically significant differences between groups were identified using the chi-square or Student's *t*-tests. Correlations between the PDMP level and continuous variables were assessed using multivariable linear regression analyses. Receiver operating characteristics curve analysis was used to estimate an optimal cutoff value for biomarkers. Overall survival (OS) was defined as the time from initial diagnosis to the time of death from any cause, or the date the patient was last known to be alive. Disease-free survival (DFS) was measured from the date of diagnosis until the date of disease recurrence or death, or until the date the patient was last known to be disease free. Univariate analyses of OS were performed using

the Kaplan-Meier product-limit method with the log-rank test and the Cox proportional hazards model. The 95% confidence interval (CI) for the survival rate was calculated using Greenwood's method. The Brookmeyer and Crowley method was used to calculate the 95% CI of the median survival time. P-values less than 0.05 were considered statistically significant. All analyses were performed using the StatFlex program (ver. 6).

RESULTS

Clinical characteristics of the study subjects

All subjects were aged 21-82 years. A total of 147 NSCLC and 35 control (bronchial asthma or chronic obstructive pulmonary disease) patients were recruited. Of the 147 NSCLC patients, 102 had a smoking history while the remaining 45 had never smoked. Table 1 details the histological classification of tumors from NSCLC patients. The performance status (PS) was 0-2 in 112 patients and 3 or 4 in 35 patients. Eleven patients had stage IIIb disease, whereas 131 patients had stage IV disease. One hundred and twenty-nine patients had received at least one regimen of systemic chemotherapy, whereas 18 patients had received best supportive care alone.

Levels of various biomarkers in NSCLC

The levels of IL-6 and MCP-1 were not significantly different between the two groups (Table 2). However, levels of RANTES, sVCAM-1, sE-selectin, HMGB1, Ang-2, VEGF, PAI-1 and PDMP were higher in NSCLC patients compared with control patients (Table 2). Notably, the difference in PDMP levels exhibited the strongest statistical significance ($p < 0.001$; Table 2).

Variable analysis of various biomarkers

We next used univariate and multivariate regression analyses to investigate associations between 15 variables and PDMP concentration in NSCLC patients (Table 3). Univariate analyses revealed that PLT, RANTES, sVCAM-1, sE-selectin HMGB1,

Table 1. Clinical characteristics of the study subjects.

Age, years: Median (range)	**Patients (n = 147)**		**Controls (n = 35)**	
	69 (33-85)		**63 (34-79)**	
	n	**%**	**n**	**%**
Sex (males)	100	68.0	22	62.9
Smokers	102	69.4	17	48.6
Non-smokers	45	6.8	18	51.4
Performance status				
0 - 2	112	76.2		
3 - 4	35	23.8		
Stage				
IIIb	11	7.5		
IV	131	89.1		
Cell type				
Adenocarcinoma	88	59.9		
Squamous cell carcinoma	41	27.9		
Large cell carcinoma	11	7.5		
Unknown type	7	4.8		

Table 2. Levels of soluble factors and microparticles.

Cytokine/factor	Control	NSCLC	p value
n	35	147	
IL-6 (pg/ml)	12.2 ± 8.9	14.7 ± 19.3	N.S.
MCP-1 (pg/ml)	464 ± 162	526 ± 123	N.S.
RANTES (ng/ml)	56.9 ± 10.7	74.9 ± 23.6	< 0.05
sVCAM-1 (ng/ml)	725 ± 128	1,102 ± 399	< 0.05
sE-selectin (ng/ml)	66.3 ± 11.4	87.2 ± 20.5	< 0.05
HMGB1 (ng/ml)	7.4 ± 1.6	13.6 ± 3.1	< 0.01
Ang-2 (pg/ml)	1,088 ± 396	1,572 ± 736	< 0.05
VEGF (pg/ml)	419 ± 86	582 ± 129	< 0.05
PAI-1 (ng/ml)	14.2 ± 4.5	24.4 ± 5.5	< 0.01
PDMP (U/ml)	13.7 ± 3.9	31.6 ± 7.1	< 0.001

IL-6: interleukin-6; MCP-1: monocyte chemotactant protein-1; RANTES: regulated on activation normally T-cell expressed and secreted; sVCAM-1: soluble vascular cell adhesion molecule-1; sE-selectin: soluble E-selectin; HMGB1: high mobility group box 1; Ang-2: angiopoietin-2; VEGF: vascular endothelial growth factor; PAI-1: plasminogen activator inhibitor-1; PDMP: platelet-derived microparticle. The p values are for control vs. NSCLC. Data are shown as means ± S.D. N.S.: not significant

VEGF and PAI-1 were significantly associated with PDMP. Using multivariate analyses, HMGB1 and PAI-1 levels were also found to be significantly correlated with PDMP levels (Table 3).

Survival analysis using PDMP, HMGB1, and PAI-1

Univariate analyses identified significantly increased OS in female patients ($p = 0.002$), those who had never smoked ($p = 0.075$), those with a PS of 0-2 ($p < 0.001$) and those with non-squamous cell carcinoma ($p = 0.001$) (Table 4).

We also analyzed the contribution of newly-designed risk factors (NDRF) to OS or DFS. NDRF classification was determined based on levels of PDMP, HMGB1 and PAI-1. Table 5 indicates that the number of NSCLC patients with high levels of PDMP, HMGB1 and PAI-1 was 63, 34 and 41, respectively. NDRFs were classified as 0-3 according to the following definitions. NDRF0: patients with low levels of PDMP, HMGB1 and PAI-1; NDRF1: patients with one of PDMP, HMGB1, or PAI-1 elevated; NDRF2: patients with two of PDMP, HMGB1, or PAI-1 elevated; and NDRF3: patients with PDMP, HMGB1 and PAI-1 all elevated (Table 5).

We next evaluated the association between levels of PDMP, HMGB1 and PAI-1 and patient outcomes using a Kaplan-Meier survival analysis in the derivation cohort to determine the individual prognostic power of each biomarker. NDRF3 was significantly correlated with a poor prognosis ($p < 0.05$ for both OS and DFS; Figures 1 and 2). NDRF2 was associated with an unfavorable OS ($p < 0.05$; Figure 1), but was not associated with DFS (Figure 2). Neither NDRF1 nor NDFR0 was correlated with either OS or DFS (Figures 1 and 2).

DISCUSSION

Multiple studies have attempted to identify prognostic factors or molecular biomarkers to predict the likelihood of lung cancer metastases or recurrence. Presently useful prognostic factors include disease staging, performance status, histology, sex and age [28-30]. Additionally, recent efforts have focused on identifying potential endothelial, hematological, or inflammatory

Table 3. Multiregression analysis on PDMP in NSCLC.

Analysis	Univariate		Multivariate	
	β	p value	β	p value
Age (years)	0.2193	0.08397		
Sex (men)	- 0.0956	0.32413		
Hb (g/dl)	0.1766	0.12377		
WBC ($\times 10^2/\mu l$)	- 0.0233	0.28361		
PLT ($\times 10^4/\mu l$)	0.3197	0.04833*	0.2545	0.06392
CRP (mg/dL)	0.2391	0.08655		
IL-6 (pg/ml)	0.2198	0.09214		
MCP-1 (pg/ml)	0.1967	0.11638		
RANTES (ng/ml)	0.4145	0.00338*	0.2521	0.05972
sVCAM-1 (ng/ml)	0.3917	0.00995*	0.2317	0.09751
sE-selectin (ng/ml)	0.3862	0.00815*	0.2749	0.08335
HMGB1 (ng/ml)	0.4275	0.00182*	0.3136	0.04137*
Ang-2 (pg/ml)	0.2394	0.08643		
VEGF (pg/ml)	0.3279	0.04521*	0.2357	0.09751
PAI-1 (ng/ml)	0.6138	0.00009*	0.5576	0.08335

Abbreviations: Hb, hemoglobin; WBC, white blood cell; PLT, platelet; CRP, c-reactive protein; for rest of the abbreviations see table 2.
β: standardized progression coefficients; *: $p < 0.05$, statistically significant.

Table 4. Univariate analysis of overall survival.

Variable	MST (months)	p value
Age, ≦70 y vs. ≧71 y	12.9 vs. 9.9	0.0756
Female vs. male	15.4 vs. 10.2	0.0029
Non-smokers vs. smoker	16.4 vs. 11.1	0.0095
ECOG PS 0-2 vs. 3-4	13.8 vs. 4.9	< 0.0001
Non-sq vs. sq.	13.1 vs. 9.5	0.0014
Stage IIIb vs. IV	13.6 vs. 10.1	0.1158

MST: median survival time; PS: performance status.

biomarkers for NSCLC [7, 11, 12, 13, 31]. We found that levels of RANTES, sVCAM-1, sE-selectin, HMGB1, Ang-2, VEGF, PAI-1 and PDMP were higher in NSCLC patients than in control individuals. Some of these biomarkers have been previously associated with NSCLC prognosis [6, 12, 32-34]. Here, we concentrated on PDMP as the difference in PDMP levels between control patients and NSCLC patients was the strongest of the differences observed.

PDMP is a platelet-related biomarker with procoagulant activity and contributes to thrombosis

Table 5. NDRF classification using PDMP, HMGB1 and PAI-1.

		n
Marker		
PDMP	low (< 30.55)	84
	high (≥ 30.55)	63
HMGB1	low (< 12.95)	113
	high (≥ 12.95)	34
PAI-1	low (< 23.61)	106
	high (≥ 23.61)	41
New index by above markers		
NDRF0: no high level of above markers		73
NDRF1: no fewer than one high level of above markers		31
NDRF2: no fewer than two high level of above markers		22
NDRF3: all high level of above markers		21

NDRF: newly-designed risk factor.

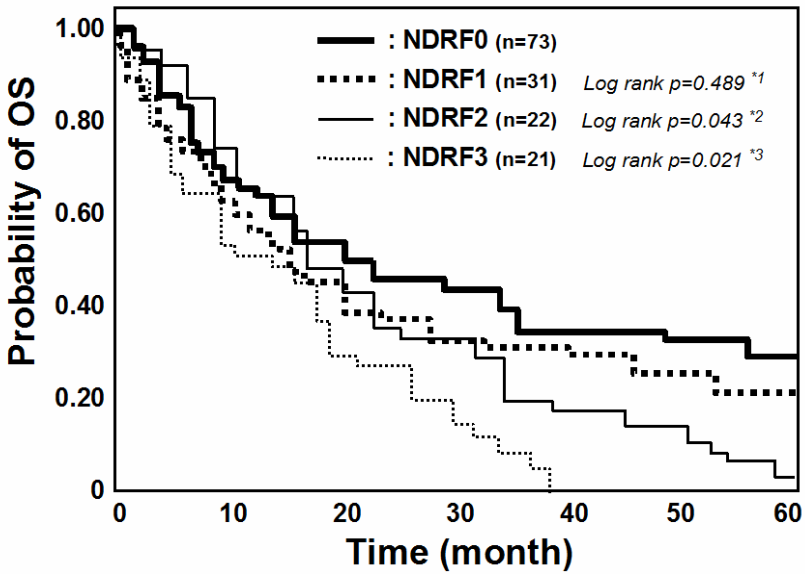

Figure 1. Kaplan-Meier curves for overall survival (OS) of the patients according to NDRF.
NDRF: newly-designed risk factor.

formation and atherosclerosis [23-25]. Several associations between platelet-based parameters and NSCLC progression have been previously reported [7, 8, 30]. Inagaki *et al*. [7] reported that the MPV: platelet count ratio is closely associated with survival in patients with advanced NSCLC. Also, Kumagai *et al*. [8] reported that a low MPV predicted an unfavorable prognosis following curative resection of NSCLC. Furthermore, Zhang *et al*. [30] conducted a meta-analysis and demonstrated that an elevated platelet count confers a poor prognosis for patients with lung cancer.

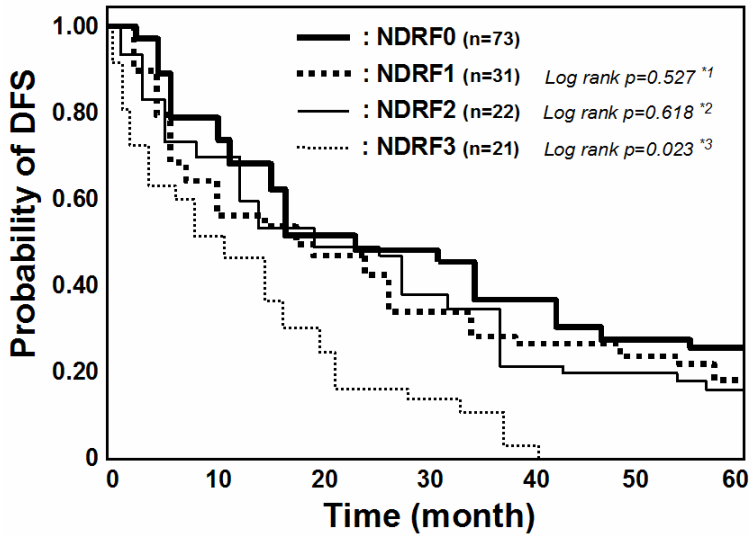

Figure 2. Kaplan-Meier curves for disease-free survival (DFS) of the patients according to NDRF. NDRF: newly-designed risk factor.

Therefore, we investigated whether PDMP or PDMP-associated biomarkers were associated with NSCLC prognosis. We investigated 15 variables and their associations with PDMP in NSCLC patients by multivariable analysis. Levels of HMGB1 and PAI-1 were found to be significantly correlated with PDMP level.

HMGB1 has been previously reported as a potential prognostic factor for NSCLC [6, 32-34]. Naumnik *et al*. [6] identified increased levels of HMGB1 in patients with advanced NSCLC undergoing chemotherapy. However, they concluded that HMGB1 concentration did not influence survival time following NSCLC treatment because there was no significant difference in HMGB1 levels before and after chemotherapy. Contrastingly, Wang *et al*. [32] reported that HMGB1 was highly expressed in NSCLC and may be valuable as a prognostic and predictive marker for NSCLC. Therefore, the relevance of HMGB1 for NSCLC prognosis is controversial.

Su *et al*. [34] reported that high PAI-1 expression in NSCLC correlated with a poor prognosis. However, they also observed that this effect of PAI-1 is dependent on PAI-2. Therefore, the individual relevance of these markers for NSCLC prognosis remains unclear. Nevertheless, PAI-1, and/or HMGB1, levels may be important prognostic factors for lung cancer [33, 35].

We found that NSCLC patients with combined high levels of PDMP, HMGB1 and PAI-1 had a poor prognosis. A previous report has described a significant elevation of PDMP in lung cancer [36]. However, we did not identify any individual effects of PDMP, HMGB1 or PAI-1 for NSCLC prognosis.

The level of endothelial cell-derived microparticles (EDMP) has also been identified as a prognostic biomarker for NSCLC [11, 12, 36]. Fleitas *et al*. [11] reported that circulating levels of EDMP and circulating endothelial cells correlate with prognosis, and could be useful prognostic markers for patients with advanced NSCLC. Consistently, Wang *et al*. [12] suggested that circulating EDMPs may be a useful biomarker predictive of 1-year mortality in end-stage NSCLC patients. Furthermore, Tseng *et al*. [36] reported that of all MPs investigated, only an increased level of EDMP was significantly associated with lung cancer. Unfortunately, we could not measure EDMP in the present study. Therefore, it remains unknown whether high levels of PDMP, HMGB1 and PAI-1 in NSCLC patients are associated with the level of EDMP.

We propose that HMGB1 plays an important role in the relationships between HMGB1, PDMP and PAI-1. HMGB1 is a nuclear protein that binds to nucleosomes and promotes DNA bending [37].

However, HMGB1 released from intracellular stores into the blood stream also plays a crucial role in the cellular response to tissue damage [38] HMGB1 expression is detectable in multiple immune and inflammatory diseases [39], and it is sequestered by thrombomodulin *in vivo* [40]. Moreover, HMGB1 stimulates toll-like receptor (TLR) and receptor for advanced glycation endproducts (RAGE). Therefore, HMGB1 can activate platelets through TLR4. Activation of TLR or RAGE on endothelial cells or platelets stimulates release of PAI-1 and PDMP into the circulation and promotes vasculopathy. Therefore, the presence of HMGB1, PDMP and PAI-1 could contribute to vascular complications such as thrombosis formation. As many individuals with cancer are in a hypercoagulable state, the elevated risk of thrombosis conferred by hypercoagulativity increases patient morbidity and mortality [14].

CONCLUSION

Our findings have two potential implications. First, we have shown that a combined increase in levels of HMGB1, PDMP and PAI-1 is related to NSCLC prognosis. Second, we have described how vascular complications may result from increased levels of these biomarkers to contribute to poor prognosis for NSCLC. Nevertheless, our study has some limitations. We were unable to determine whether any relationship between EDMP and the three biomarkers exists. Additionally, we did not investigate how different therapeutic strategies affect the utility of the prognostic markers identified. Further confirmation of our observations in prospective studies is necessary.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests regarding the publication of this paper.

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