Lower Osteopontin Plasma Levels Are Associated With Superior Outcomes in Advanced Non–Small-Cell Lung Cancer Patients Receiving Platinum-Based Chemotherapy: SWOG Study S0003

Philip C. Mack, Mary W. Redman, Kari Chansky, Stephen K. Williamson, Nichole C. Farneth, Primo N. Lara Jr, Wilbur A. Franklin, Quynh-Thu Le, John J. Crowley, and David R. Gandara

From the University of California, Davis Cancer Center, Sacramento; Stanford University, Stanford; Veterans Administration of Northern California, Martinez, CA; Fred Hutchinson Cancer Research Center; Cancer Research and Biostatistics, Southwest Oncology Group, Seattle, WA; University of Kansas Medical Center, Kansas City, KS; and University of Colorado Health Sciences Center, Denver, CO.

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Corresponding author: Philip C. Mack, PhD, Division of Hematology and Oncology, University of California, Davis Cancer Center, 4501 X St, Ste 3016, Sacramento, CA 95817; e-mail: pcmack@ucdavis.edu.

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ABSTRACT

Purpose

S0003 was a phase III trial of carboplatin/paclitaxel with or without the hypoxic cytotoxin tirapazamine in patients with advanced or metastatic non–small-cell lung cancer (NSCLC). We investigated the relationship between clinical outcomes and plasma levels of the hypoxia-associated protein osteopontin (OPN) in patients on this protocol.

Patients and Methods

Baseline plasma was obtained from 172 patients. In 56 patients, sequential plasma was obtained after one or two cycles. Concentrations of OPN, as well as plasminogen activator inhibitor-1 (PAI-1) and vascular endothelial growth factor (VEGF), were measured using enzyme-linked immunosorbent assay. Tumor expression of OPN was assessed by immunohistochemistry in 61 matched archival specimens.

Results

Patients with lower OPN levels (below the median) had a significantly superior overall survival compared with patients with higher levels, regardless of treatment arm (hazard ratio [HR] = 0.60, $P = .002$). A similar correlation was observed for progression-free survival (HR = 0.69, $P = .02$). When examined as a continuous variable, OPN maintained its significant association with both progression-free (HR = 1.05, $P = .01$) and overall survival (HR = 1.09, $P < .0001$). Patients with lower plasma OPN levels were significantly more likely to have tumor response ($P = .03$). No differences were observed between treatment arms. Tumor OPN levels did not correlate with patient outcomes or with plasma levels. No associations were observed between patient outcomes and VEGF or PAI-1 levels; however, plasma concentrations of these markers were significantly interrelated ($P < .0001$) and significantly decreased after treatment ($P = .0002$ and $P = .03$, respectively).

Conclusion

Pretreatment plasma levels of OPN are significantly associated with patient response, progression-free survival, and overall survival in chemotherapy-treated patients with advanced NSCLC.

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INTRODUCTION

Lung cancer is the most common cause of cancer-related death for both men and women in the United States, accounting for more deaths than prostate, breast, and colon cancer combined.¹ Almost 80% of patients are categorized as having non–small-cell lung cancer (NSCLC). Most NSCLC patients have either locally advanced or metastatic disease at the time of diagnosis and are, therefore, generally incurable. Despite recent evidence of therapeutic progress, currently available chemotherapy regimens provide only a modest in-

crease in overall survival and are associated with considerable toxicity.²⁻⁴ Furthermore, at present, selection of chemotherapy is empiric. Identification of prognostic and/or predictive biomarkers for chemotherapy outcomes would enhance therapeutic decision making.⁵ Because the diagnosis of NSCLC is increasingly reliant on fine-needle aspiration, providing only a small cytologic specimen, discovery of plasma-related biomarkers would be particularly valuable.

Osteopontin (OPN) is a phosphorylated acidic glycoprotein with a diverse range of biologic activities.^{6,7} Secreted OPN interacts with members of the

integrin family and variants of CD44, stimulating a variety of downstream processes associated with tumor progression or cellular transformation.⁷⁻¹⁰ OPN induces expression of urokinase-type plasminogen activator and increases cell migration and adhesion, contributing to cellular transformation and metastasis.¹¹⁻¹⁴ Furthermore, OPN levels increase in the presence of tumor hypoxia, a condition associated with both chemotherapy and radiotherapy resistance.^{15,16}

Tumor or plasma levels of OPN have previously been associated with poor outcome or an aggressive phenotype in a variety of malignancies, including renal, breast, prostate, gastric, esophageal, and head and neck carcinomas.¹⁷⁻²² Abnormal OPN levels have been well documented in lung cancer.²³⁻²⁷ In NSCLC, elevated tumor OPN mRNA or protein expression has been correlated with shortened survival in patients with early-stage disease.^{26,28,29} Moreover, plasma levels of OPN are more commonly increased in patients with higher stage disease.³⁰ In patients with pleural mesothelioma, Pass et al³¹ documented significantly increased OPN blood levels, suggesting use of this biomarker as a diagnostic tool to identify individuals with early disease after exposure to asbestos.

We hypothesized that plasma levels of OPN and two other biomarkers associated with tumor hypoxia and early progression, plasminogen activator inhibitor-1 (PAI-1) and vascular endothelial growth factor (VEGF), would correlate with patient outcomes in

S0003, a Southwest Oncology Group trial of carboplatin/paclitaxel with or without tirapazamine (TPZ). Here, we report results demonstrating that OPN concentrations were significantly associated with patient response, progression-free survival, and overall survival, independent of treatment arm.

PATIENTS AND METHODS

Study Population and Sample Collection

Peripheral-blood specimens were obtained from 172 consenting patients of 396 registered patients with histologically or cytologically confirmed stage IIIB (positive pleural effusion) or stage IV disease (no brain metastases) enrolled onto S0003.³² No significant differences were detected between patients with marker analysis and those without (Table 1). Although there is no evidence that the subset of patients analyzed for markers was not a random sample of the parent trial, it is nonetheless possible that there were systemic differences that could lead to bias in estimation of associations. Blood specimens were collected in 10-mL EDTA tubes at baseline and after the first and/or second cycles of therapy in consenting patients remaining on therapy. Plasma was isolated and stored in aliquots at -80°C .

Plasma Concentrations of OPN, VEGF, and PAI-1

Specimens were analyzed by enzyme-linked immunosorbent assay using commercially available kits following the manufacturer's instructions. PAI-1

Table 1. Patient Characteristics Based on Whether Marker Analysis Was Conducted

Characteristic	Has Marker Data				P
	No (n = 224)		Yes (n = 172)		
	No. of Patients	%	No. of Patients	%	
Treatment arm					.60
No tirapazamine	114	51	83	48	
Tirapazamine	110	49	89	52	
Age at random assignment, years					.98
< 65	126	56	97	56	
≥ 65	98	44	75	44	
Sex					.17
Female	88	39	56	33	
Male	136	61	116	67	
Histology					.96
Squamous	188	84	144	84	
Nonsquamous	36	16	28	16	
Stage					.42
III	30	13	28	16	
IV	194	87	144	84	
No. of metastatic sites					.07
None	31	11	30	14	
Single	71	33	66	40	
Multiple	122	56	76	46	
Performance status					.24
0	67	30	61	35	
> 0	157	70	111	65	
Weight loss					.81
< 5%	142	63	111	65	
≥ 5%	82	37	61	35	

NOTE. A comparison of the 172 patients from the S0003 trial for whom plasma was available for analysis v 224 patients with no available plasma revealed no statistically significant differences between these populations.

Fig 1. Survival by baseline osteopontin (OPN) plasma level. (A) Survival (Kaplan-Meier) plot showing patients dichotomized at greater than or less than the median plasma concentration of OPN (598 ng/mL). Patients with higher OPN levels (blue) had significantly worse overall survival compared with patients with lower levels (yellow; $P = .0016$). (B) Patients were subdivided into quartiles based on OPN plasma levels. Patients with increased plasma OPN concentrations performed significantly worse ($P = .001$).

Imulysse Kits were obtained from Biopool International (Ventura, CA), VEGF kits were obtained from BioSource International, Inc (Camarillo, CA), and OPN kits were obtained from Assay Designs Inc (Ann Arbor, MI). The PAI-1 kit recognizes active and latent PAI-1, as well as urokinase-type plasminogen activator/PAI-1 complexes with less efficiency. The VEGF kit recognizes the VEGF-165 form. The OPN assay recognizes the native and thrombin-cleaved forms of OPN, independent of phosphorylation state. Analyses were performed in duplicate.

Immunohistochemical Analysis of Tumor OPN

Immunohistochemical staining was performed using a manual scoring algorithm that evaluates both staining intensity and percent positivity.³³ Intensity was graded on a scale from 0 to 4+, and the percentage of tumor cells with each level of staining was summed, yielding a final score ranging from 0 to 400, as previously described.³³

Statistics

The data analysis for this article was generated using SAS/STAT software, Version 9.1.3 (SAS Institute, Cary, NC). Spearman's nonparametric correlation coefficient was used to assess correlations between OPN in tumor and plasma, PAI-1, and VEGF levels. Logistic regression was used to obtain odds ratio estimates and CIs for the associations with response. Survival distribution differences were assessed with the log-rank statistic, and a Cox proportional hazards model was used to obtain hazard ratio (HR) estimates and CIs. The Wilcoxon signed rank test was used to assess change in marker values from before to after treatment. A significance level of $P = .05$ was used.

RESULTS

Baseline Plasma Levels

Patient plasma specimens were evaluated for levels of OPN ($n = 156$), PAI-1 ($n = 153$), and VEGF ($n = 169$). Median baseline plasma levels were 592 ng/mL (interquartile range, 331 to 890 ng/mL) for OPN, 73 ng/mL (interquartile range, 24 to 155 ng/mL) for PAI-1, and 80 pg/mL (interquartile range, 13 to 231 pg/mL) for VEGF. No correlations were observed between basal OPN levels and either PAI-1 or VEGF ($P = .67$ and $P = .49$, respectively). However, PAI-1 and VEGF were significantly interrelated such that concordance in levels of both markers was common. ($r = 0.72$; $P < .0001$). OPN levels were not associated with number of metastatic sites, age, sex, stage of disease (IIIB v IV), squamous versus nonsquamous histology, or weight loss ($> 5\%$ v $\leq 5\%$). Performance status of more than 0 versus 0 was associated with higher levels of OPN ($P = .04$).

Plasma Levels of OPN, but Not VEGF or PAI-1, Are Prognostic for Patient Outcomes

The relationship between baseline levels of OPN, PAI-1, and VEGF and patient overall and progression-free survival was investigated. Figure 1A shows the overall survival of all patients stratified at the median plasma level of OPN. Patients below the median had a significantly better survival compared with patients above the median ($P = .002$). The relationship between OPN and survival was generally linear.

Distribution of baseline OPN plasma levels into quartiles demonstrated that levels in the lower quartiles were associated with significantly longer survival times, with median overall survival time for patients in the lowest quartile nearly double that of patients in the highest quartile (Fig 1B; $P = .002$). When examined as a continuous variable, OPN maintained a highly significant association with both progression-free survival and overall survival ($P = .01$ and $P < .0001$, respectively). Table 2 lists the HRs and P values for OPN, PAI-1, and VEGF for progression-free survival and overall survival as dichotomized at the median or analyzed as a continuous variable (plasma levels per increase by 100 units/mL). Neither VEGF nor PAI-1 was informative for these patient outcomes.

Patients were dichotomized as partial or complete responders versus those with progressive or stable disease. Patients who responded had lower basal levels of OPN compared with nonresponding patients ($P = .04$). For patients with a baseline plasma sample available, 33% responded to therapy. The frequency of response differed by OPN status such that 40% of patients with OPN levels less than the median responded to treatment, whereas only 25% of patients with OPN greater than the median responded to treatment ($P = .03$; Table 3). No such association was observed for either PAI-1 or VEGF.

PAI-1 and VEGF Plasma Levels Decrease Significantly After Treatment

Post-treatment plasma levels of PAI-1, VEGF, and OPN were measured in 56 patients after one or two cycles of therapy. In this subset of patients, median OPN levels were nonsignificantly reduced from 597 to 424 ng/mL ($P = .07$; Table 4). In contrast, both PAI-1 and VEGF levels showed significant reductions ($P = .0002$ and $P = .03$,

Table 2. Relationship Between Basal Plasma Levels and Patient Survival Dichotomized at the Median Value or Analyzed as a Continuous Variable

Marker	Progression-Free Survival			Overall Survival		
	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Dichotomized at the median						
OPN	0.69	0.50 to 0.95	.01	0.60	0.43 to 0.84	.002
PAI-1	0.98	0.71 to 1.35	.89	1.22	0.88 to 1.70	.23
VEGF	1.10	0.81 to 1.50	.54	1.23	0.90 to 1.68	.19
Continuous variable						
OPN	1.05	1.01 to 1.09	.02	1.09	1.04 to 1.13	< .0001
PAI-1	0.97	0.88 to 1.06	.49	0.97	0.88 to 1.07	.56
VEGF	1.03	0.99 to 1.07	.10	1.02	0.98 to 1.06	.40

NOTE. Patients with OPN levels less than the median had significantly superior overall and progression-free survival rates. For analysis as a continuous variable, the HR is calculated as the risk associated with an increase in protein concentration of 100 units/mL for each marker. For every 100-ng/mL increase in OPN plasma levels, risk of death increases by approximately 5%. No significant associations were observed between PAI-1 and VEGF and patient survival.

Abbreviations: HR, hazard ratio; OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; VEGF, vascular endothelial growth factor.

Table 3. Percentage of Responders as Correlated With Plasma Marker Expression

Marker	% of Responders		Odds Ratio	95% CI	P
	< Median	> Median			
OPN	40	25	2.2	1.1 to 4.3	.03
PAI-1	30	37	0.70	0.35 to 1.4	.31
VEGF	27	38	0.61	0.31 to 1.2	.15

NOTE. Patients with lower OPN levels are significantly more likely to have responded to treatment.

Abbreviations: OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; VEGF, vascular endothelial growth factor.

respectively). Changes in OPN, PAI-1, or VEGF levels were not significantly associated with subsequent progression-free survival ($P = .36$, $P = .40$, and $P = .28$, respectively) or overall survival ($P = .9$, $P = .39$, and $P = .24$, respectively). For patients with a greater than 10% decline in VEGF after one or two cycles of therapy, 19 (54%) of 35 patients were responders compared with five (29%) of 17 patients whose levels increased or remained static ($P = .09$). Tumor response was not associated with change in either OPN or PAI-1 levels ($P = .47$ for OPN, $P = .48$ for PAI-1). Change in OPN levels did not differ significantly between the treatment arms ($P = .75$). Levels declined by 152 ng/mL in the control arm and by 109 ng/mL in the experimental arm.

Tumor Protein Levels of OPN and Patient Outcome

Tumor expression of OPN was assessed by immunohistochemistry using a manual quantitative scoring methodology that produces a score ranging from 0 to 400. Representative examples of high and low OPN staining are shown in Figure 2. The average score for 53 assessable tumor sections was 240. Eleven patients had a score of 400, indicating highly intense staining in 100% of tumor cells. Thirteen patients had tumors scoring less than 200, indicating light or moderate staining. One patient had no OPN tumor expression. Whether evaluated by immunohistochemistry score or intensity of staining, there was no statistical association between OPN tumor expression and patient outcomes. Patients responded at equivalent rates whether tumor OPN score was greater than or less than the median ($P = .41$). Similarly, no correlations were observed between tumor OPN and progression-free survival ($P = .93$) or overall survival ($P = .75$).

Associations between tumor expression of OPN and plasma concentrations of OPN, PAI-1, and VEGF were explored. OPN concentrations in tumor and plasma were not significantly correlated, with a correlation coefficient of 0.25 ($P = .19$). An inverse

Fig 2. Immunohistochemical staining of tumor osteopontin (OPN). Archival tumor specimens from 61 patients were evaluated by immunohistochemistry using a manual quantitative scoring method derived from staining intensity and percent positivity (range, 0 to 400). Shown are representative samples with a (A) high and a (B) low score. The majority of tumors had sharply elevated OPN expression relative to adjacent normal tissue, with a mean score of 254.

association between PAI-1 plasma levels and tumor OPN was noted ($\rho = -0.45$, $P = .008$).

DISCUSSION

In the present study, we hypothesized that levels of the hypoxia-related proteins OPN, PAI-1, and VEGF would correlate with clinical outcomes in advanced-stage NSCLC patients enrolled onto S0003. Hypoxia, as defined by subphysiologic levels of oxygen, is present in the majority of human tumors and is prevalent in NSCLC.³⁴⁻³⁶ Tumor hypoxia is associated with amplified signaling for angiogenesis, resistance to chemotherapy and radiotherapy, and increased potential for tumor progression and metastasis.³⁷⁻⁴⁰ For example, cisplatin had little or no cytotoxic activity against hypoxic tumor cells in a preclinical model despite killing a large proportion of nonhypoxic cells.⁴¹ In addition, malignant cells subjected to hypoxia are induced to transcribe a discrete set of genes, including the genes coding for OPN, PAI-1, and VEGF.^{42,43} Hypoxia may then exert selection pressure on tumor populations by promoting angiogenesis, *p53* mutations, and resistance to apoptosis. Combined, these elements result in a worse overall survival for patients with extensive tumor hypoxia.⁴⁴ Efforts to combat tumor hypoxia include the use of anticancer agents activated

Table 4. Post-Treatment Changes in Marker Expression

Marker	Median Value		Median % Decrease	P	% of Patients With Decreasing Value	
	Before Treatment	During Treatment			> 10%	> 30%
OPN	597 ng/mL	424 ng/mL	19	.07	55	47
PAI-1	109 ng/mL	56 ng/mL	44	.0002	64	57
VEGF	103 pg/mL	56 pg/mL	52	.03	67	58

NOTE. The absolute value and median percent decrease in plasma level of each marker are shown after one or two cycles of therapy. Both PAI-1 and VEGF were significantly decreased during treatment. Also shown are the percentages of patients with a > 10% or 30% decrease in plasma concentrations.

Abbreviations: OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; VEGF, vascular endothelial growth factor.

by low oxygen conditions.^{45,46} TPZ, a novel benzotriazine compound, is a bioreductive agent that, in low-oxygen environments, is reduced to form cytotoxic free radicals.⁴⁵ Preclinical studies showed that TPZ was two to three orders of magnitude more toxic to hypoxic cells than to oxygenated cells.^{47,48} In a phase III trial (Cisplatin Tirapazamine in Subjects With Advanced Previously Untreated Non–Small-Cell Lung Tumors 1), the addition of TPZ to cisplatin was found to significantly prolong survival in patients with advanced NSCLC; however, a second phase III study comparing cisplatin and etoposide with cisplatin and TPZ found no improvement in response rates or survival.⁴⁹ S0003 was closed early after an interim analysis showed that the addition of TPZ would not result in improved outcomes.³²

Here, we report that low plasma concentrations of OPN were a highly significant marker of superior patient outcome, regardless of treatment arm. When stratified at the median, patients with low OPN values had a significantly better overall survival compared with patients with values greater than the median (HR = 0.60, $P = .002$). The relationship between OPN and survival was generally linear, such that the higher the OPN plasma concentration was, the shorter the patient survival. OPN levels were also associated with response to therapy, with the odds of responding among patients with OPN levels greater than the median approximately half the odds of patients with OPN levels less than the median ($P = .02$). Failure of OPN as a biomarker to identify patients who benefited from TPZ may reflect lack of efficacy of TPZ, alternative mechanisms for OPN induction, or inability of the current study sample size to discern differences. Although PAI-1 and VEGF levels were inter-related and declined after therapy, neither was associated with patient outcomes.

No significant association between OPN plasma levels and tumor expression as measured by immunohistochemistry could be demonstrated. One explanation for this observation is that nonmalignant cells may be contributing to plasma OPN concentrations. It has long been established that alternative components of the tumor microenvironment can play significant roles in tumor progression.⁵⁰ In particular, monocytes that are recruited to malignancies differentiate into macrophages.⁵¹ Tumor hypoxia can greatly influence the cytokine expression profiles of these tumor-associated macrophages, inducing expression of proangiogenic and mitogenic factors.^{52,53} Cheng et al⁵⁴ determined that OPN secreted by macrophages can restore metastatic potential in OPN-silenced SK-Hep-1 cells when grown in cocultures. By immunohistochemistry, we observed elevated OPN staining in a variety of nontumor cells within the tumor environment. Positive entities included stroma, lymphocytes, and alveolar macrophages. Secreted OPN from these nonmalignant, tumor-associated cells likely contributes to tumor growth, survival, invasiveness, and metastatic

potential. Therefore, plasma levels of OPN may prove to be more predictive than tumor expression as a marker of poor prognosis because it would capture both tumor-specific and tumor-associated (nontumor) production of OPN.

In summary, we found that, in chemotherapy-treated patients with advanced NSCLC, low plasma levels of OPN were significantly associated with improved clinical outcomes. Patients with lower levels had better response rates and higher overall and progression-free survival rates. OPN plasma level may prove to have utility as a prognostic biomarker in chemotherapy-treated patients with unresectable NSCLC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

Conception and design: Philip C. Mack, Quynh-Thu Le, David R. Gandara

Financial support: Philip C. Mack, David R. Gandara

Administrative support: Philip C. Mack

Provision of study materials or patients: Philip C. Mack, Mary W.

Redman, Kari Chansky, Stephen K. Williamson, David R. Gandara

Collection and assembly of data: Philip C. Mack, Mary W. Redman, Kari Chansky, Stephen K. Williamson, Nichole C. Farneth, Wilbur A. Franklin, John J. Crowley, David R. Gandara

Data analysis and interpretation: Philip C. Mack, Mary W. Redman, Kari Chansky, Primo N. Lara Jr, Wilbur A. Franklin, Quynh-Thu Le, John J. Crowley, David R. Gandara

Manuscript writing: Philip C. Mack, Mary W. Redman, Primo N. Lara Jr, Wilbur A. Franklin, Quynh-Thu Le, David R. Gandara

Final approval of manuscript: Philip C. Mack, Mary W. Redman, Kari Chansky, Stephen K. Williamson, Nichole C. Farneth, Primo N. Lara Jr, Wilbur A. Franklin, Quynh-Thu Le, John J. Crowley, David R. Gandara

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