An Evaluation of Tumor Oxygenation and Gene Expression in Patients with Early Stage Non–Small Cell Lung Cancers

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Abstract

Background: To directly assess tumor oxygenation in resectable non–small cell lung cancers (NSCLC) and to correlate tumor pO_2 and the selected gene and protein expression to treatment outcomes.

Methods: Twenty patients with resectable NSCLC were enrolled. Intraoperative measurements of normal lung and tumor pO_2 were done with the Eppendorf polarographic electrode. All patients had plasma osteopontin measurements by ELISA. Carbonic anhydrase-IX (CA IX) staining of tumor sections was done in the majority of patients ($n = 16$), as was gene expression profiling ($n = 12$) using cDNA microarrays. Tumor pO_2 was correlated with CA IX staining, osteopontin levels, and treatment outcomes.

Results: The median tumor pO_2 ranged from 0.7 to 46 mm Hg (median, 16.6) and was lower than normal lung pO_2 in all but one patient. Because both variables were affected by the completeness of lung deflation during measurement, we used the ratio of tumor/normal lung (T/L) pO_2 as a reflection of tumor oxygenation. The median T/L pO_2 was 0.13. T/L pO_2 correlated significantly with plasma osteopontin levels ($r = 0.53$, $P = 0.02$) and CA IX expression ($P = 0.006$). Gene expression profiling showed that high CD44 expression was a predictor for relapse, which was confirmed by tissue staining of CD44 variant 6 protein. Other variables associated with the risk of relapse were T stage ($P = 0.02$), T/L pO_2 ($P = 0.04$), and osteopontin levels ($P = 0.001$).

Conclusions: Tumor hypoxia exists in resectable NSCLC and is associated with elevated expression of osteopontin and CA IX. Tumor hypoxia and elevated osteopontin levels and CD44 expression correlated with poor prognosis. A larger study is needed to confirm the prognostic significance of these factors.

Tumor hypoxia has been shown to affect the malignant progression of transformed cells and their response to therapy through the selection of tumor cells with diminished apoptotic potential and increase metastatic ability (1). Past studies have shown a strong correlation between pretreatment tumor pO_2 and tumor control and survival in patients with solid cancers (2–5). Indirect assessment of tumor hypoxia with imaging studies using hypoxia-specific markers such as [^{18}F]fluoromisonidazole and ^{60}Cu-ATSM suggest that tumor hypoxia is prevalent in non–small cell lung cancers (NSCLC; refs. 6–8). Immunohistochemical staining surrogate markers of hypoxia such as carbonic anhydrase IX (CA IX) or hypoxia-inducible factors-1α or -2α in NSCLC archival tumor tissues corroborate the prevalence of hypoxia in these tumors and its adverse prognostic effect on treatment outcomes (9–13). However, to date, there has been no study that directly assesses tumor pO_2 in patients with resectable NSCLC and correlates tumor pO_2 with intrinsic hypoxia markers and patient outcomes.

Osteopontin is a secreted phosphoglycoprotein that is induced in several solid tumors including breast, gastric, hepatocellular, and colorectal carcinomas. High levels of osteopontin protein expression, either in tumor cells or in the surrounding stroma, have been correlated with increased invasion in several solid tumors (14–17). Likewise, patients with metastatic neoplasms or with poor prognosis generally have higher plasma osteopontin levels than those without (18–20). Gene expression studies have identified osteopontin as the lead marker for colon cancer progression (21) and elevated tumor osteopontin expression is correlated with worse outcomes in node-negative breast cancers (19, 22). Data for

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Received 9/19/05; revised 11/9/05; accepted 12/30/05.

Grant support: USPHS grant CA-67166 (Q-T. Le, A.J. Giaccia), the Damon Runyon-Lilly Clinical Investigator Award (A.C. Koong) and the Stanford Cancer Council grant (Q-T. Le, C.S. Kong).

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Note: Presented at the 11th World Conference on Lung Cancer, Barcelona, Spain, July 2005.

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doi:10.1158/1078-0432.CCR-05-2049

Table 1. Patient, tumor, and treatment characteristics

Variable		No. of patients
Age	≤65	8
Median, 66 (44-82)	>65	12
Gender		
Male		10
Female		10
Smoking		
No		6
Yes		14
T Stage		
T_1		8
T_2		11
T_3		1
N Stage		
N_0		13
N_1		4
N_2		3
Stage		
I		12
II-IIIA		8*
Histology		
Adenocarcinoma		10
Squamous cell carcinomas		2
Mix		5
Bronchoalveolar carcinomas		2
Undifferentiated carcinomas		1
Tumor volume	≤10 mL	10
Median, 10.8 mL (0.1-144.4)	>10 mL	10
Median, tumor pO_2 (mm Hg)	≤10	9
Median, 16.6 (0.7-46)	>10	11
Median, lung pO_2 (mm Hg)	≤100	14
Median, 42.8 (23-656)	>100	6
Median, T/L pO_2	<0.13	9
Median, 0.13 (0.02-1.19)	≥0.13	11
Hemoglobin (g/dL)	≤13.5	10
Median, 13.5 (11.8-19.6)	>13.5	10
Plasma osteopontin (pg/mL)	<300	13
Median, 275 (147-623)	≥300	7
CA IX (n = 16)		
Negative-weak		6
Strong		10
Gene array analysis		
Yes		12
No		8
Type of surgery		
Pneumonectomy		1
Lobectomy		16
Wedge		3
Postoperative radiation		
No		14
Yes		6
Postoperative chemotherapy		
No		18
Yes		2
Relapse		
No		12
Yes		8
Relapse pattern		
Distant only		4
Distant + lung		3
Lung only		1

*There were 3 stage IIIA.

osteopontin expression is less well-established for lung cancers. One study found increased osteopontin levels in the serum of 20 patients with lung cancer (23). Another study found that strong tumoral osteopontin protein expression was associated with more advanced tumor and nodal stage (24). In addition, osteopontin plasma levels were more likely to be elevated in patients with NSCLC than in those with benign pulmonary disease or in healthy volunteers. We have previously shown that osteopontin expression was related to tumor hypoxia (20) and its mRNA expression was regulated by tumor hypoxia in head and neck cancers and in NIH-3T3 cells (25).

To further assess the role of tumor hypoxia in NSCLC, we did a prospective study to determine the effect of tumor hypoxia on outcome in patients with resectable NSCLC using the Eppendorf pO_2 histograph, CA IX staining, plasma osteopontin levels, and gene expression profiling. We found that tumor hypoxia in resectable NSCLC was associated with increased expression of osteopontin and CA IX. In addition, tumor hypoxia and elevated osteopontin and CD44 expression seemed to affect the risk of relapse in these patients.

Materials and Methods

Patients. Twenty patients with newly diagnosed early stage operable NSCLC were enrolled in this prospective study. All signed an Institutional Review Board–approved informed consent. Table 1 summarizes pertinent patient and treatment characteristics. A wedge resection was done in three patients due to preoperative poor lung function; the remainder underwent at least a lobectomy and mediastinal lymph node sampling or dissection. Most patients had pathologic T_{1-2} tumors, except for one who had a pathologic T_3 tumor due to chest wall invasion. Three patients had clinically negative but pathologically involved mediastinal nodes on nodal dissection. Adjuvant radiotherapy was given to six patients due to mediastinal nodal involvement in three patients, chest wall involvement in one patient, extensive lymphovascular and pleural involvement in one patient, and incomplete wedge resection in one patient. Adjuvant chemotherapy was given concurrently with radiotherapy for incomplete resection of an involved mediastinal node in one patient and incomplete wedge resection of the tumor in one patient. At the time of this study, adjuvant chemotherapy was not considered a standard approach for patients with early stage resected NSCLC. All patients had preoperative plasma osteopontin measurements, 16 had available lung tumor tissues for CA IX staining, and 12 had adequate frozen tumor

available for gene array analysis. The median follow up was 23 months for living patients (range, 6-55).

pO_2 measurements. All patients underwent a thoracotomy under general anesthesia. After complete deflation of the ipsilateral lung, intraoperative pO_2 measurements were recorded first from the normal lung then in the tumor using sterile techniques and a computerized histograph (Sigma Eppendorf PO_2 Histograph, Hamburg, Germany) as previously described (26). A median of 64 measurements in two to three tracks were obtained in both normal lung (range, 32-90) and tumor tissues (range, 32-120). The inspired pO_2 (FIO_2) was maintained at >50%; however, the ipsilateral lung was perfused but not ventilated. Recorded values were corrected for barometric pressure and body temperature. Data were presented in the form of median pO_2 values.

Osteopontin measurements. Plasma samples were obtained preoperatively, aliquoted and kept at −80°C until analysis. Plasma osteopontin levels were determined using an ELISA method (Assay Design, Ann Arbor, MI) as previously described (20).

CA IX and CD44 variant 6 immunohistochemical staining. Immunohistochemical staining of CA IX was done on tumor tissue sections as previously described (9). Immunoperoxidase stain for CD44 variant 6 (CD44v6; 1:50 dilution; Novocastra Laboratories, Ltd., Newcastle upon Tyne, United Kingdom) was done on 4-μmol/L-thick paraffin sections. The slides were deparaffinized in xylene then hydrated prior to antigen retrieval by microwaving in sodium citrate buffer (pH 6.0). The slides were then incubated with a peroxidase block, followed by the primary antibody. After a PBS wash, the slides were incubated with the secondary antibody and 3,3′-diaminobenzidine. The peroxidase block, secondary antibody and 3,3′-diaminobenzidine were from the DakoCytomation EnVision System (Glostrup, Denmark). After a hematoxylin counterstain (Hematoxylin 7211; Richard-Allen Scientific, Kalamazoo, MI), the slides were coverslipped. The slides were interpreted by a board-certified pathologist, who was blinded to the pO_2 data and scored as follows: negative, 0; equivocal or uninterpretable, 1; weakly positive, 2; and strongly positive, 3. Cytoplasmic and membrane staining patterns were scored separately. For the purpose of correlation with tumor pO_2 and treatment outcomes, tumors that stained strongly positive for CA IX or CD44v6 in the membrane or the cytoplasm or both locations were classified as the "strongly positive" group and those that had negative or weak staining in either or both locations were categorized as the "negative-weak" group.

Gene expression profiling. Gene expression profiling of tumor and normal lung tissues were done using the cDNA arrays from the Stanford Functional Genomic Facility as previously described (27). RNA was extracted from frozen tissues (either normal lungs or crudely dissected tumors) using Trizol. Duplicate samples were used to confirm reproducibility in half of the patients. Significance analysis of microarray (http://www-stat.stanford.edu/~tibs/SAM/) was used for supervised analysis in relation to treatment-relapse (28).

Statistical analysis. All statistical analyses were done using the Statview statistical software package (SAS Institute, Inc., Cary, NC). The Student's *t* test and simple regression method were used to determine the relationship between different clinical and biological variables including CA IX staining and plasma osteopontin levels to T/L pO_2. Freedom from relapse (FFR) and overall survival (OS) were computed with the Kaplan-Meier product limit method (29). Log-rank statistics were used to compare survival curves (30). Multivariate analysis was not done due to the small sample size.

Results

Hypoxia. Figure 1 shows the histogram of median tumor pO_2 (Fig. 1A), normal lung pO_2 (Fig. 1B), and the correlation between the two variables (Fig. 1C). The median tumor pO_2 ranged from 0.7 to 46 mm Hg (median, 16.6) and the median lung pO_2 ranged from 23 to 656 mm Hg (median, 42.8). In all but one patient, the median tumor pO_2 was lower than that of adjacent normal lung tissues. When three patients with extremely high lung pO_2 from incomplete lung deflation during Eppendorf microelectrode measurements were included (Fig. 1C), there was a statistically significant correlation between tumor and lung pO_2

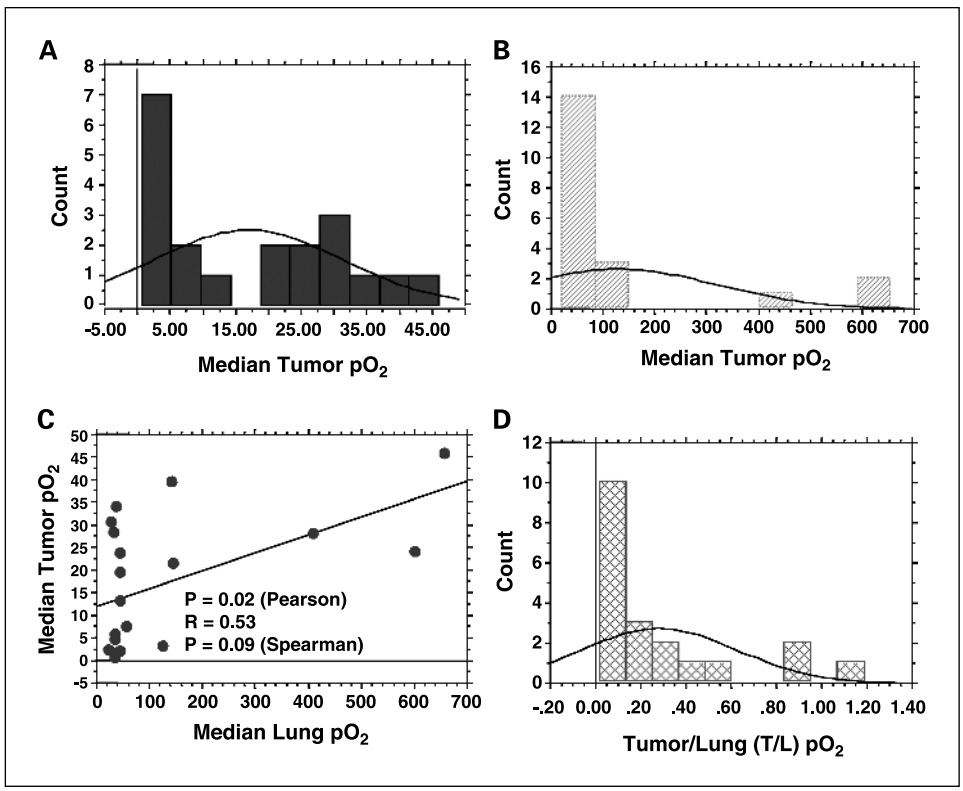

Fig. 1. *A,* histogram of median tumor pO_2. *B,* histogram of median lung pO_2. *C,* correlation between median tumor and lung pO_2. Both Spearman and Pearson correlations were reported. Spearman correlation was used to minimize the effect of the three extreme values on the correlation analysis. *D,* histogram of median tumor/lung (T/L) pO_2.

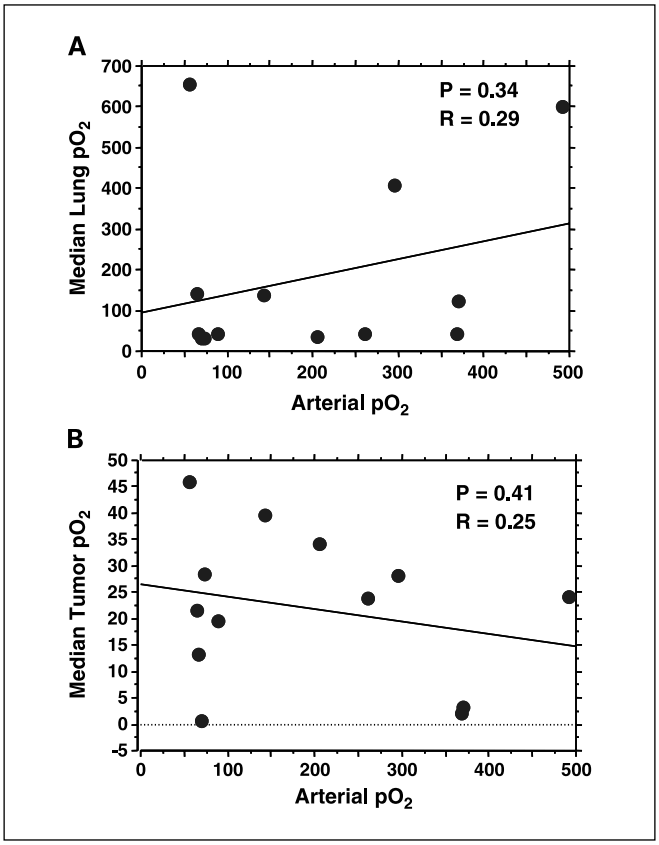

Fig. 2. *A,* correlation between arterial and lung pO_2. *B,* correlation between arterial and tumor pO_2.

(P = 0.02, Pearson's correlation). However, when Spearman correlation was used to minimize the effect of extreme values, the P values were not statistically significant. Therefore, to minimize the influence of incomplete lung deflation, we used the ratio of tumor/lung pO_2 as the expression of tumor oxygenation in these patients. Figure 1D shows the histogram of T/L pO_2. An arterial blood gas at the time of pO_2 measurement was also obtained for the first 13 patients. No correlation was noted between lung and arterial pO_2 (Fig. 2A) or tumor and arterial pO_2 (Fig. 2B). This lack of correlation persisted even when the patients with extremely high lung pO_2 were removed from the analysis. The lack of correlation was probably due to the fact that the FIO_2 was kept above the normal physiologic level of 21% during pO_2 measurement for patient safety.

Table 2 showed the correlation between different clinical and biological variables and T/L pO_2. There was a significant correlation between T/L pO_2 and CA IX staining. The mean T/L pO_2 was high (i.e., less hypoxic) at 0.56 (SE, 0.11) for tumors that stained either negatively or weakly positive for CA IX, whereas it was lower (i.e., more hypoxic) at 0.11 (SE, 0.03) for those that stained strongly positive for CA IX (P = 0.006; Fig. 3A). Both T/L pO_2 (P = 0.002) and median tumor pO_2 (P = 0.005) correlated with CA IX staining when the three patients with extremely high lung pO_2 were removed from the analysis. In addition, there was a significant inverse correlation between T/L pO_2 and plasma osteopontin levels (P = 0.04, r = 0.45) suggesting that hypoxic tumors generally had higher osteopontin levels than those that were less hypoxic (Fig. 3B). No other clinical or biological factor tested in Table 2 correlated with T/L pO_2.

Gene expression profiling. Twelve patients had available frozen tissues with adequate tumoral RNA for gene expression study. We used significance analysis of microarray statistical algorithm and supervised analysis to evaluate gene expression in relation to relapse. Of 36,453 cDNAs in the arrays, only 2,250 showed consistently adequate expression in duplicate samples for further analysis. Using these 2,250 cDNAs, the top gene, whose expression significantly correlated with relapse, was CD44. Patients with relapse had a 3.5-fold higher CD44 gene expression compared to those without relapse. To confirm the relationship between CD44 expression and relapse, we stained tumor sections with anti-CD44v6 antibody. All tumors that had elevated CD44 gene expression stained strongly positive for CD44v6 whereas most (5 of 6) of those with low CD44 gene expression had negative staining for CD44v6. As predicted by the gene expression study, strong CD44v6 protein expression correlates with FFR on univariate analysis (P = 0.03, Fig. 4A; Table 3).

Osteopontin. Because osteopontin was not present in the gene arrays that were used, we could not directly assess its gene expression with patient outcomes. However, we were able to measure osteopontin protein levels in the blood of all participating patients. Because CD44v6 is a surface receptor of osteopontin and has been shown to be induced by osteopontin (31), we evaluated the expression of osteopontin and CD44v6. We found that osteopontin levels significantly correlated

Table 2. Correlation between different variables and tumor/lung pO_2

Variable	*R* value or median (SE)	*P*
Age	0.27	0.24
Gender		
Male (n = 10)	0.42 (0.14)	0.07
Female (n = 10)	0.14 (0.04)	
Smoking Status		
Nonsmoker (n = 6)	0.21 (0.90)	0.57
Smoker (n = 14)	0.31 (0.10)	
Tumor volume	0.22	0.34
Tstage		
T_1 (n = 8)	0.39 (0.12)	0.26
T_{2-3} (n = 12)	0.21 (0.10)	
N stage		
N_0 (n = 13)	0.33 (0.10)	0.44
N_{1-2} (n = 7)	0.20 (0.12)	
Stage		
I (n = 12)	0.35 (0.11)	0.29
II-III (n = 8)	0.18 (0.11)	
Histology		
Squamous (n = 5)	0.22 (0.17)	0.65
Nonsquamous	0.30 (0.09)	
Hemoglobin	0.06	0.80
Arterial pO_2 (n = 13)	0.25	0.41
Arterial pCO_2 (n = 13)	0.04	0.89
Osteopontin	0.45	0.04
CA IX		
Negative-weak (n = 6)	0.56 (0.18)	0.006
Strong (n = 10)	0.11 (0.03)	

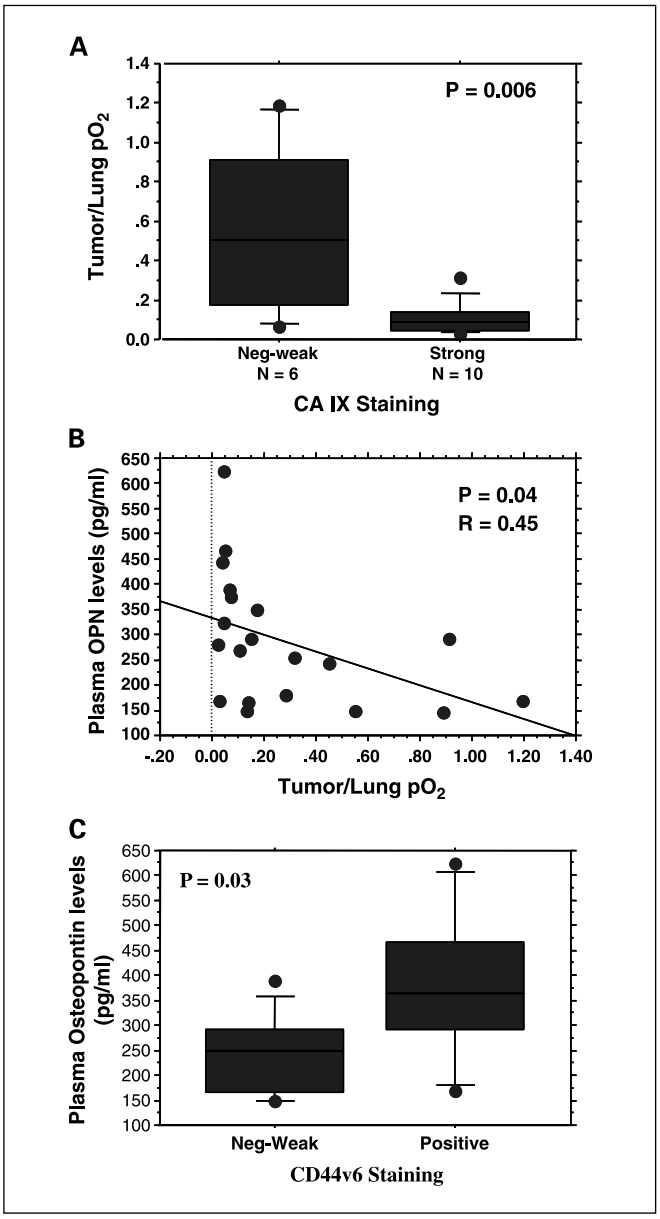

Fig. 3. *A,* correlation between T/L pO_2 and CA IX staining. Patients were grouped into negative-weak staining group versus strong-staining group based on CA IX cytoplasmic and membrane expression. *B,* correlation between T/L pO_2 and plasma osteopontin levels. *C,* correlation between osteopontin plasma level and CD44v6 staining.

with CD44v6 staining (P = 0.03, Fig. 3C). In addition, high osteopontin plasma levels also predicted for a higher risk of relapse both as a dichotomous (P = 0.001, Fig. 4B) and a continuous variable (P = 0.005).

Freedom from relapse. At the latest follow up, eight patients had relapsed. The first site of relapse was distant only in four patients, distant and thoracic in three patients, and thoracic in only one patient. The 1- and 2-year FFR rates for all patients were 72% and 59%, respectively. In addition to CD44v6 and osteopontin, we also analyzed other variables for risk of relapse. These included gender, T stage, N stage, histology, T/L pO_2, and CA IX staining. Table 3 shows the results of univariate analysis for FFR. High T stage (P = 0.03, Fig. 4C) and low T/L pO_2 (more hypoxic tumors, P = 0.04 for all patients, P = 0.03 when patients with extremely high lung pO_2 were removed; Fig. 4D) also correlated with increased risk of relapse. Multivariate analysis was not done due to the small number of patients.

Overall survival. At the latest follow up, nine patients had died: six from tumor relapse, one from suicide, one from respiratory failure 2 months postoperative, and one from *Pneumocystis carinii* pneumonia. The 1- and 2-year OS rates were 90% and 52%, respectively. Table 3 shows the univariate analysis for predictors for OS. Only N stage (P = 0.001, Fig. 5A) and T/L pO_2 (P = 0.02 for all patients, P = 0.04 when patients with extremely high lung pO_2 were removed; Fig. 5B) achieved statistical significance. T stage and osteopontin levels [both as dichotomous and continuous variables (P = 0.09)] nearly reached statistical significance. Multivariate analysis was again not done here due to the small number of patients.

Discussion

Tumor hypoxia is a common phenomenon in solid tumors. Direct pO_2 measurements documenting hypoxia in sarcomas, head and neck, cervical, pancreatic, and prostate carcinomas have been previously published (2–5, 26, 32, 33). No such direct evidence has been documented in early stage NSCLC because these tumors are often deeply situated and inaccessible to direct measurements without triggering severe complications such as pneumothorax. Therefore, direct assessment of tumor pO_2 can only be done intraoperatively for these tumors. This is the first report of direct pO_2 measurements in patients undergoing surgery for early resectable NSCLC. Because all intraoperative measurements were done during unilateral lung ventilation, we were unable to adjust inhaled pO_2 to ambient room air levels (21%) without compromising patient safety. Inhaled pO_2 was therefore kept at levels >50% in all patients. Although we found no correlation between arterial and tumor pO_2, we did note a correlation between tumor and lung pO_2 and that both variables could be affected by the completeness of lung deflation during the measurements. The three patients who had incomplete lung deflation had much higher lung and generally higher tumor pO_2 than the remaining patients. Therefore, we believe that the ratio of tumor to lung pO_2 (T/L pO_2) is a better reflection of tumor oxygenation than the median tumor pO_2 alone in these patients. Using this ratio, we found a strong correlation between tumor oxygenation and the protein expression of CA IX, which has been shown by several investigators to be an endogenous surrogate marker for hypoxia and prognosis in NSCLC (9, 10, 12, 13). Specific to early stage NSCLC, Kim et al. studied CA IX expression in 74 patients with resected NSCLC and noted an association between CA IX staining, tumor necrosis, and hypoxia-inducible factor-1α expression (12, 13). More importantly, high CA IX expression was an independent prognostic factor for disease-free survival on multivariate analysis (13). In our smaller study, T/L pO_2, but not CA IX expression, was associated with the risk of tumor relapse and death. The discrepancy between this study and that by Kim et al. could be explained by the small patient sample size in

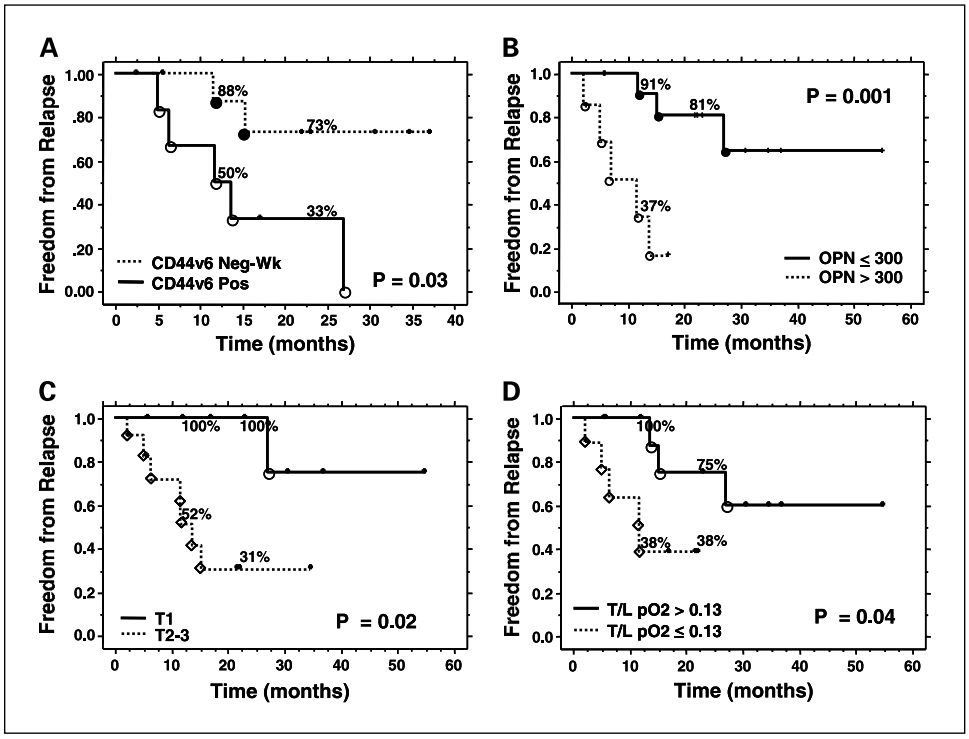

Fig. 4. *A,* FFR by CD44v6 immunohistochemical staining. *B,* FFR by plasma osteopontin levels. *C,* FFR byTstage. *D,* FFR byT/L pO_2.

Table 3. Prognostic factors for FFR and OS on univariate analysis

Variable	No. of patients	FFR		OS	
		2-y FFR (%)	*P*	2-y OS (%)	*P*
Gender					
Male	10	56	0.59	56	0.68
Female	10	61		50	
Tstage					
1	8	100	0.02	88	0.06
2-3	12	31		31	
N stage					
0	13	66	0.13	78	0.001
1-2	7	40		14	
Histology					
Squamous cell carcinomas	5	40	0.07	55	0.47
Others	15	66		50	
Tumor/lung pO_2					
‹0.13	9	38	0.04	79	0.02
›0.13	11	75		17	
CA IX					
Negative-weak	6	50	0.47	83	0.37
Strong	10	48		47	
Osteopontin					
‹300	13	81	0.001*	64	0.09*
≥300	7	17	0.005†	26	0.09†
CD44v6					
Negative-weak	10	73	0.03	64	0.92
Strong	6	33		60	

*Dichotomous.
†Continuous.

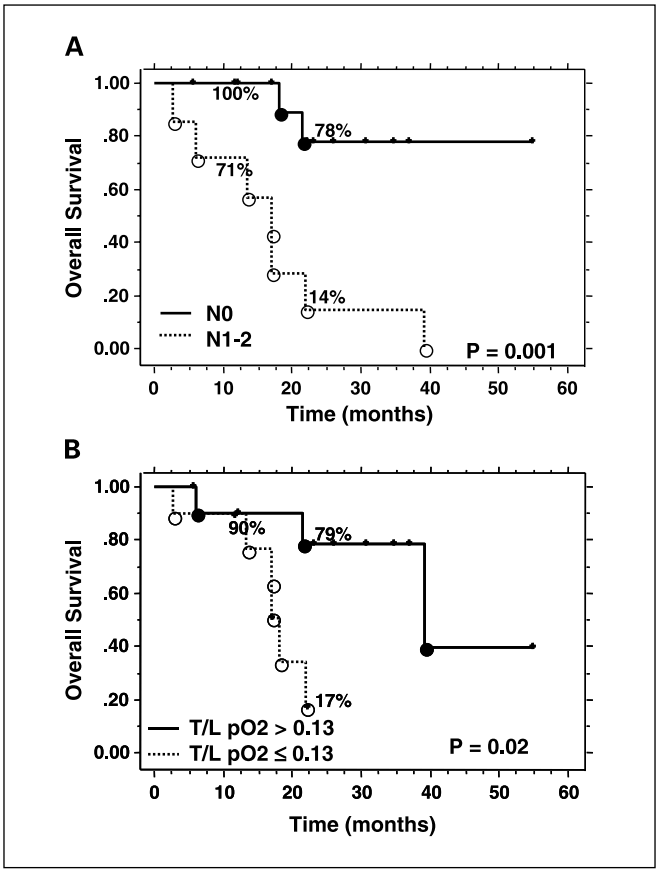

Fig. 5. *A,* OS by N stage. *B,* OS by T/L pO_2.

our study and potential sampling error as tumor pO_2 was measured throughout the tumor, whereas CA IX staining was done only on a single tumor section.

We have previously shown that osteopontin is a hypoxia-related secreted protein in head and neck cancers (20), and that its gene expression is induced by hypoxia in several cell types (25). Osteopontin gene expression has been shown to be elevated in patients with pulmonary squamous cell carcinomas in a tissue expression profiling study using cDNA subtraction and microarray analysis (34). Plasma osteopontin levels have also been shown to be elevated in patients with NSCLC in comparison to those with benign lung disease or healthy volunteers (23, 24). Furthermore, elevated tumor osteopontin protein levels were associated with more advanced tumor stage and a higher risk of nodal metastasis in these NSCLC patients (24). To further investigate the role of osteopontin in NSCLC, we measured plasma osteopontin levels in the 20 early stage NSCLC patients reported here. Similar to our observation in head and neck cancer patients, there was a significant correlation between plasma osteopontin levels and T/L pO_2; patients with more hypoxic tumors generally had higher plasma osteopontin levels. In addition, elevated plasma osteopontin levels correlated with the risk of relapse both as a dichotomous and as a continuous variable, supporting the concept that osteopontin is important for tumor aggressiveness in NSCLC.

Osteopontin has multiple receptor binding domains and could interact with several cell surface receptors (35, 36). One such receptor is CD44, which is a family of transmembrane glycoproteins that are expressed in hematopoietic and epithelial cells and are associated with diverse physiologic functions including cell-cell and cell-matrix interactions (35). CD44 exists in multiple isoforms produced by alternative mRNA splicing and CD44v6, a known marker of type II pneumocytes, is the primary isoform that interacts with osteopontin (36). Osteopontin has been shown to increase the expression of CD44v6 (31) and interactions between these two molecules have been implicated in cellular transformation, tumor growth, tumor cell adhesion, and host-immune cell recognition (31, 35, 37–39). In addition, elevated expression of CD44 mRNA has been implicated with worse outcome in breast cancers (34). Engagement of CD44 with lung cancer cells has been shown to reduce Fas expression and Fas-mediated apoptosis, resulting in less susceptibility of the cells to CTL-mediated cytotoxicity (40). These data suggest that the CD44-osteopontin interaction could be an important factor for tumor cell aggressiveness in NSCLC. In this small study, using cDNA microarray, we identified CD44 overexpression as a prognostic factor for relapse. We confirmed the gene expression data by staining tumor sections with CD44v6 and found a correlation between gene and protein expression as well as protein staining and FFR. In addition, there was a strong correlation between CD44 expression and plasma osteopontin levels, suggesting that these two proteins may act in concert to promote tumor aggressiveness in these patients.

In summary, we have done a comprehensive study of tumor hypoxia in patients with respectable NSCLC. We found that tumor hypoxia exists in these patients and is associated with elevated expression of osteopontin and CA IX. Tumor hypoxia and the elevated expression of osteopontin and CD44 proteins also seem to affect prognosis in these patients. A major drawback of this study is its small sample size and the heterogeneous histologies and stages included. These data should provide the stimulus for a larger clinical study to confirm the prognostic significance of these biological factors.

Acknowledgments

We thank Neeta Nair for her administrative support in preparing and submitting the manuscript, and Alithea Morasca for her assistance in operating the Eppendorf pO_2 histograph.

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