

Carbonic Anhydrase IX in Early-Stage Non–Small Cell Lung Cancer

Seok Jin Kim,¹ Zahid N. Rabbani,²
Robin T. Vollmer,³ Ernst-Gilbert Schreiber,⁴
Egbert Oosterwijk,⁵ Mark W. Dewhirst,²
Zeljko Vujaskovic,² and Michael J. Kelley¹

¹Department of Medicine, Thoracic Oncology Program, Duke University Medical Center and Durham Veterans Affairs Hospital, Durham, North Carolina; ²Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina; ³Department of Pathology, Duke University Medical Center and Durham Veterans Affairs Hospital, Durham, Veterans Affairs; ⁴Pulmonary Division, University of Utah, Salt Lake City, Utah; ⁵Department of Urology, University Hospital Nijmegen, Nijmegen, the Netherlands

ABSTRACT

Purpose: Tumor hypoxia is associated with poor prognosis and increased tumor aggressiveness. Carbonic anhydrase (CA) IX, an endogenous marker for tumor hypoxia, catalyzes the hydration of carbon dioxide into carbonic acid and contributes to the pH regulation of tumor cells. Therefore, CA IX might allow tumors to acclimate to a hypoxic microenvironment, promoting tumor cell proliferation. We hypothesized that CA IX expression is related to tumor cell proliferation and poor disease-free survival in patients with early-stage non–small-cell lung cancer (NSCLC).

Experimental Design: CA IX expression was measured in 75 resected NSCLC tumors to assess prognostic implications for disease-free survival. The relationship of CA IX expression with microvessel density (MVD) and proliferation (Ki-67) index was assessed via colocalization analysis.

Results: All patients had operable NSCLC (stage I, 58; stage II, 17). CA IX expression was present in 54 (72%) of 75 patients and was associated with tumor necrosis ($P < 0.05$). CA IX-positive tumor areas showed greater cell proliferation as measured by Ki-67 index ($P < 0.05$) and less MVD ($P < 0.05$) than did CA IX-negative areas in colocalization analysis. The percentage of CA IX-positive tumor cells was significantly related to postoperative recurrence and poor disease-free survival ($P < 0.05$). Ki-67 index and pathologic

stage were also independent prognostic factors for worse disease-free survival ($P < 0.05$).

Conclusions: CA IX expression of tumor cells may be an indicator for poor disease-free survival in early-stage NSCLC.

INTRODUCTION

The tumor microenvironment, which includes the interaction between proliferating tumor cells and angiogenesis, has been shown to be relevant in tumor progression toward a more malignant phenotype (1). Thus, assessment of tumor microenvironment may provide information about patients with high risk of recurrence and poor prognosis. Tumor hypoxia has been considered as a functional marker of the tumor microenvironment and could result from an imbalance between tumor cell proliferation and angiogenesis (2). Although it has long been known as a cause of treatment resistance, tumor hypoxia is also associated with increased tumor cell survival and aggressiveness via clonal selection of cells with increased malignant potential (3–5). In addition, hypoxia itself has been reported to lead to genetic alterations promoting tumor cell growth and angiogenesis (6, 7).

Carbonic anhydrase (CA) IX, an endogenous marker for tumor hypoxia, is a transmembrane enzyme (8) that catalyzes the reversible hydration of carbon dioxide into carbonic acid (9). Although the role of CA IX in tumor progression is still unclear, CA IX might help maintain a normal pH in tumor cells under hypoxia, allowing tumors to acclimate to a hypoxic microenvironment, which may permit continued tumor cell proliferation away from blood vessels (10, 11). Transfection of CA IX promotes cell proliferation (12, 13), whereas blockade of CA IX results in increased cell death under hypoxic conditions (14). CA IX expression has been reported to have prognostic significance in several tumor types including head and neck, and kidney (15, 16). However, the value of this marker is controversial, because other studies have not shown significant correlations between CA IX and outcome in cervical nor in head and neck cancers (17, 18).

In non-small cell lung cancer (NSCLC), a relationship between CA IX expression and overall survival has been reported (19, 20). However, its relation with postoperative recurrence and disease-free survival has not been assessed. Considering the substantial incidence of postoperative recurrence in early-stage (stage I-II) NSCLC (21), a relationship of CA IX with recurrence might identify a patient group with high risk of recurrence. Furthermore, analyzing the interrelationship of CA IX, cell proliferation, and angiogenesis should provide a better understanding of the tumor microenvironment and complementary information relevant for treatment. Therefore, we examined the relationship between CA IX expression and both disease-free survival and overall survival in early-stage NSCLC. To gain additional insights into the role of CA IX function in malignant

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Requests for reprints: Michael J. Kelley, Hematology/Oncology (111G), 508 Fulton Street, Durham, NC 27705. Phone: (919) 286-0411, extension 7326; Fax: (919) 286-6896; E-mail: kelley@m@duke.edu.

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disease, we also examined the spatial relationship of its expression with tumor cell proliferation and tumor angiogenesis.

MATERIALS AND METHODS

Study Population

Seventy-five patients with previously untreated NSCLCs from the Durham Veterans Affairs Hospital were included in this study. Morphologic classification of the carcinomas was assigned according to the WHO criteria; 38 patients had squamous cell carcinomas, 27 adenocarcinomas, 6 large-cell carcinomas, and 4 untyped non-small-cell carcinomas. All of the patients were staged at the time of surgery following the guidelines of the American Joint Committee on Cancer Staging (21), and all of the patients had mediastinal lymph node evaluation at the time of surgery by mediastinoscopy and/or mediastinal lymph node sampling as currently recommended for early-stage NSCLC (22). Fifty-eight patients had stage I tumors and 17 patients had stage II tumors. All patients had surgical resection of the primary tumor. Six patients received adjuvant or neoadjuvant therapy. Two patients received adjuvant chemotherapy (T2N0 and T2N1) on clinical trials. One patient received neoadjuvant combined chemotherapy/radiation (T2N1) on a clinical trial. Three patients received postoperative radiation therapy for positive margins (one T1N0 and two T1N1). All patients were male, and their ages ranged from 53 to 86 years (median, 67 years). The median follow-up of surviving patients at the time of analysis was 35 months (range, 4–84 months). Follow-up data were obtained from medical records. Survival times were measured from the date of surgery.

Immunohistochemistry

Immunohistochemistry for CA IX, CD31, and Ki-67 was performed with consecutive 5- μ m serial sections of formalin-fixed, paraffin-embedded tissues placed onto positively charged glass slides with a single-staining procedure. The Ki-67 index was assessed by one investigator (R. T. V) and evaluation of CA IX and CD31 expression was performed by two investigators (S. J. K. and Z. N. R.). All of the interpretations of immunohistochemistry were performed without knowledge of clinical outcome.

CA IX. For the detection of CA IX, we used the anti-CA IX mouse monoclonal antibody clone M75 (gift from Dr. Oosterwijk, Department of Urology, University Hospital Nijmegen, the Netherlands). Tissue sections were dewaxed and rehydrated. After peroxidase was quenched with methanol and 3% hydrogen peroxide for 10 minutes, microwave antigen retrieval was done twice on high power for 5 minutes each. After blocking with 10% donkey serum, the slides were incubated with the primary antibody (3 μ g/mL) overnight at 4°C and were washed with Tris-buffered saline (TBS). Positive control was human cervical squamous cell carcinoma tissue, which has previously been established as positive for CA IX (18, 23). Simultaneous incubation of slides in which primary antibody was omitted served as negative control. Biotinylated donkey antimouse antibody (1:1000, v/v) was applied for 30 minutes at room temperature, followed by application of ABC kit (Vector Lab, Inc., Burlingame, CA). Slides were again washed in TBS, and color was developed by 5 minutes' incubation in 3,3'-diaminobenzidine

(DAB) solution. Slides were counterstained with hematoxylin. To assess variability of immunostaining, we included slides from a strongly staining case in each batch of 20 tumor samples. Two investigators (S. J. K. and Z. N. R.) evaluated all slides independently, and differences between the two observers were resolved by consensus. After the slides were scanned at low magnification ($\times 40$), three to seven areas (per case) of maximum CA IX expression were selected. The degree of positive CA IX staining on the section was assessed at high magnification ($\times 200$) based on the semiquantitative scale of 0 to 4, and the percentage of tumor cells staining for CA IX was measured at low magnification ($\times 40$). The mean value of the examined fields was the final value. The CA IX score was derived from the product of the percentage of tumor cells staining for CA IX and the average intensity of that staining.

CD31. Microvessel counting was used to assess angiogenesis. The anti-CD31 mouse monoclonal antibody clone JC70A (Dako, Copenhagen, Denmark) was used to stain microvessels. Primary antibody (dilution, 1:20) was applied at room temperature for 60 minutes, and the slides were stained by the avidin-biotin method, as described above. After the slides were scanned at low magnification ($\times 40$), four areas (per case) of high vascularization were chosen for microvessel counting. Microvessel density (MVD) was expressed as the number of vessels per field. The mean value from four fields was recorded as the MVD for each tumor.

Ki-67. The anti-Ki-67 rabbit monoclonal antibody, NCL Ki67 (Novocastra Laboratory Ltd., Newcastle, United Kingdom) was applied (dilution, 1:500) overnight at 4°C, and slides were stained by the avidin-biotin method, as described above. Tumor cells were considered positive for the Ki-67 antigen if there was intranuclear DAB staining. After the slides were scanned at low magnification ($\times 40$), the cells with positively stained nuclei were counted in a maximum of 10 fields at a magnification of $\times 650$. Four hundred to 2,000 tumor cells were counted, and Ki-67 index was calculated as the percentage of positive cells per positive and negative cells.

Assessment of Tumor Necrosis

Tumor necrosis was assessed on both CA IX-stained sections and hematoxylin and eosin (H&E)-stained sections. The extent of necrosis was assessed at low magnification ($\times 40$), and cases were divided into three groups according to the extent of necrosis as follows: minimal necrosis group, with necrosis in less than 5% of the optical fields; severe necrosis group, with necrosis in more than 15%; and moderate necrosis group, consisting of all other cases.

Colocalization of Markers

Because serial sections from each tumor had been studied for all three markers, the regional correlation of the three markers was investigated. Eighty-two tumor areas having strong CA IX expression were chosen from 30 strongly CA IX-positive cases ($>2+$) and were compared with 31 tumor areas without CA IX expression from the same cases. In corresponding areas, microvessels and positively stained nuclei for Ki-67 were counted.

Statistical Analysis

The Fisher's Exact Test was applied to assess the association between categorical variables. Coefficient of correlation (r) between expressions of markers was calculated with the Spearman Rank Test. Disease-free survival and overall survival curves were calculated with the Kaplan–Meier Method and were compared by the Log-Rank test. The Cox proportional hazards regression model was used for multivariate analyses. All of the statistical analyses were performed with a statistical software package, SPSS, version 10.0 (SPSS Inc., Chicago, IL). Statistical significance was defined as P values less than 0.05. All P values were two-sided.

RESULTS

CA IX Expression, Percentage of CA IX-Positive Tumor Cells, and CA IX Score. CA IX expression was analyzed with three methods: (a) mean staining intensity, on a grading scale of 0 to 4; (b) the percentage of positive cells; and (c) the product of the staining intensity and percentage of positive cells (CA IX score). Cases were classified as positive if the mean staining intensity was grade 1 or greater. Of the 75 cases analyzed, 54 (72%) cases were positive for CA IX. The staining pattern was characteristic membranous and cytoplasmic localization. The median percentage of CA IX-positive tumor cells was 7.5%, and the median CA IX score was 11.4. We examined the correlation of these three CA IX variables with pathologic variables in our patients with NSCLC (Table 1).

All three variables were higher in squamous cell carcinomas than in adenocarcinomas ($P < 0.05$). For example, 31 (82%) of 38 of the squamous cell carcinomas were positive for CA IX, but only 15 (56%) of 27 adenocarcinomas were positive. Tumor necrosis was strongly associated with CA IX expression ($P < 0.01$). Forty-one (89.1%) of 46 cases with moderate or severe necrosis were positive for CA IX. Both the CA IX score

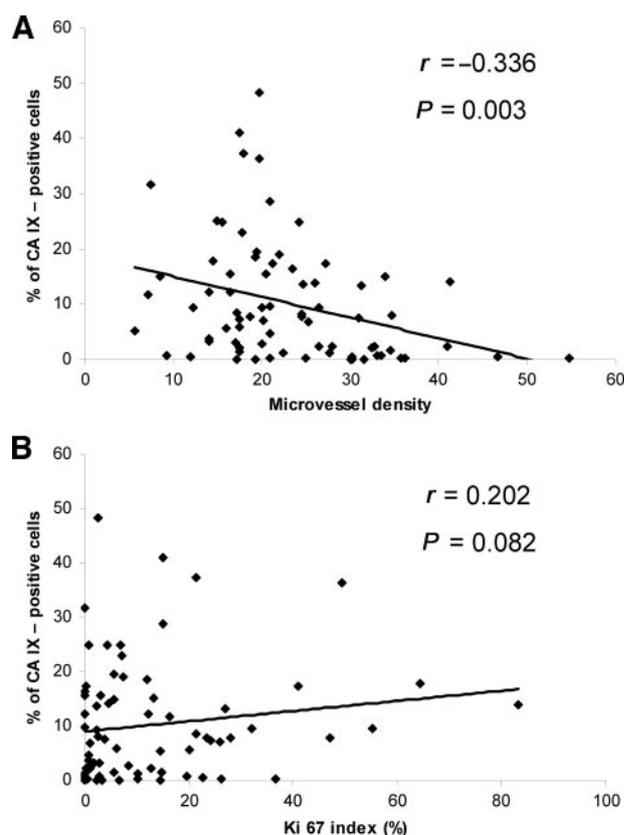


Fig. 1 Correlations between the percentage of CA IX-positive cells, MVD, and Ki-67 index. In A, the mean MVD tended to decrease as the percentage of CA IX-positive tumor cells increased ($r = -0.336$, $P = 0.003$), suggesting that less vascularized areas are more hypoxic. In B, Ki-67 index showed a weak correlation with the percentage of CA IX-positive tumor cells, but it was marginally significant ($r = 0.202$; $P = 0.082$). Spearman Correlation Test was used.

Table 1 Correlation of CA IX variables with pathologic variables

Variables	CA IX expression			% of CA IX-positive cells			CA IX score		
	Negative	Positive	P value *	<7.5	≥7.5	P value *	<11.4	≥11.4	P value *
Histology			0.02 †			0.02 †			0.01 †
Squamous cell carcinoma ($n = 38$)	7	31		15	23		14	24	
Adenocarcinoma ($n = 27$)	13	14		19	8		20	7	
Large-cell carcinoma ($n = 6$)	1	5		2	4		2	4	
Others ($n = 4$)	1	3		1	3		1	3	
T stage ‡			0.29			0.83			0.83
T1 ($n = 33$)	8	25		17	16		17	16	
T2 ($n = 39$)	14	25		19	20		19	20	
T3 ($n = 3$)		3		1	2		1	2	
N stage ‡			0.43			0.31			0.74
N0 ($n = 66$)	18	48		31	35		32	34	
N1 ($n = 9$)	4	5		6	3		5	4	
Stage ‡			0.99			0.18			0.79
I ($n = 58$)	17	41		26	32		28	30	
II ($n = 17$)	5	12		11	6		9	8	
Tumor necrosis			<0.01			0.10			0.03
Minimal ($n = 29$)	17	12		18	11		19	10	
Moderate to severe ($n = 46$)	5	41		19	27		18	28	

* Fisher's Exact test was applied to the association of CA IX, MVD, and Ki-67 index with clinicopathologic variables.

† The association of three CA IX variables with squamous cell carcinoma and adenocarcinoma was analyzed by Fisher's exact test.

‡ Pathologic stage was used.

and the percentage of CA IX positive tumor cells trended toward higher values in tumors with necrosis, but the differences were not statistically significant ($P = 0.10$). There was no significant association between CA IX variables and other pathologic conditions such as tumor size, nodal involvement, or pathologic stage.

Association of CA IX with Angiogenesis and Cell Proliferation. To assess interrelationships between CA IX and angiogenesis or cell proliferation, the CA IX variable values were compared with MVD and Ki-67 index, respectively, with the Spearman Rank Test (Fig. 1). All three CA IX variables showed statistically significant, but weak, inverse correlations with MVD. Comparisons were also made by examining average Ki-67 and MVD values, grouped above or below the median CA IX variable values. For example, in the group with high percentages of CA IX-positive tumor cells ($>7.5\%$), the mean Ki-67 index was significantly greater than the low percentage group (16.7 ± 20.3 versus 8.1 ± 9.6 ; $P = 0.02$). The other two CA IX variables were not significantly correlated with Ki-67.

The comparisons above were done without regard to spatial localization of the variables. To examine the spatial correlation between angiogenesis and cell proliferation, we quantified colocalization of expression of the three markers: CA IX, CD31, and Ki-67. In the subgroup of 30 cases with high levels of CA IX staining, 82 regions were assessed. Qualitatively, areas staining positive for CA IX showed fewer microvessels than negatively stained areas (Fig. 2). Overall, microvessels were not observed in 74 (90%) of these sections. By comparison, all 31 areas without CA IX expression contained microvessels ($P < 0.01$).

Ki-67 index was positively correlated with CA IX expression in the colocalization analysis. Seventy-one of 82 CA IX-positive areas showed Ki-67 expression levels greater than 5.6%. Further, Ki-67 expression greater than 5.6% was found more frequently in CA IX-positive than in CA IX-negative areas (71 (87%) of 82 versus 19 (61%) of 31; $P < 0.01$). These findings suggest that CA IX expression is correlated with increased oxygen consumption induced by relatively high tumor cell proliferation (24).

CA IX and Postoperative Recurrence. Among the 20 cases with tumor recurrence, 18 were local recurrences and two were at distant sites. The percentage of CA IX-positive tumor cells was significantly correlated with recurrence ($P = 0.02$; Table 2). Fifteen cases (39.5%) had tumor recurrence among the 38 patients with high percentage of CA IX-positive tumor cells ($>7.5\%$). CA IX score and Ki-67 index trended higher in those cases that recurred, although this did not reach statistical significance ($P = 0.06$, for both). However, CA IX expression, as a binary variable (positive or negative), was not associated with recurrence ($P = 0.39$). Patients with pathologic stage II had a nonsignificant higher recurrence rate [7 (41.1%) of 17] compared with patients with stage I [13 (22.4%) of 58; $P = 0.21$]. To evaluate the association of MVD and recurrence, we dichotomized based on the value of MVD of 15, 20, 25, and 30%, and median value. All of them failed to show statistically significant relationship with postoperative recurrence. The presence of tumor necrosis was also not related to recurrence.

Relationship of CA IX to Disease-Free Survival and Overall Survival. Thirty-two (43%) of the 75 patients died during the follow-up period (median follow-up, 35 months). The

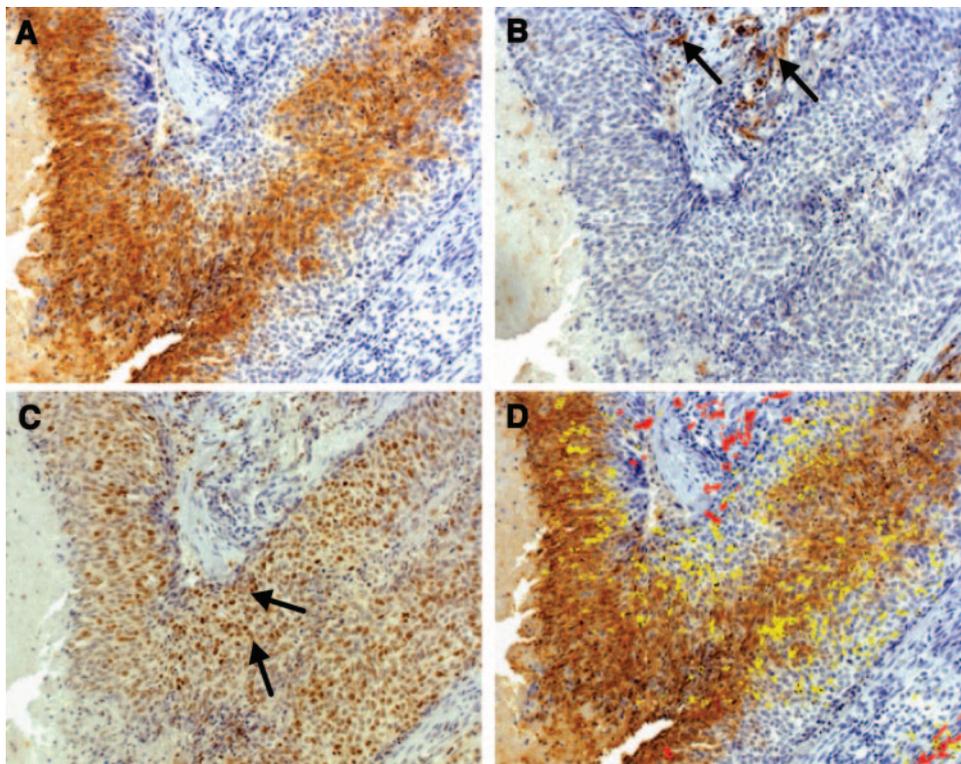


Fig. 2 Colocalization of CA IX, MVD (CD31), and Ki-67 in a representative NSCLC tumor. Immunohistochemistry is performed on serial tissue sections as described in the Materials and Methods ($\times 200$). **A**, brown-colored cytoplasmic staining, CA IX expression. **B**, CD31 staining, microvessels (black arrows); **C**, brown-colored nuclei (black arrows), Ki-67 expression. CD31 staining in **B** (red) and Ki-67 expression in **C** (yellow) are overlaid on the CA IX staining in **D**.

Table 2 Relationship of CA IX variables and other pathologic variables to disease recurrence

Variables	Nonrecurrence	Recurrence	P value *
Stage †			
I	45	13	0.21
II	10	7	
CA IX expression			
Negative	18	4	0.39
Positive	37	16	
% of CA IX-positive cells			
<7.5%	32	5	0.02
≥7.5%	23	15	
CA IX score			
<11.4	31	6	0.06
≥11.4	24	14	
Microvessel density			
<21	26	10	0.99
≥21	29	10	
Ki-67 index (%)			
<5.6	31	6	0.06
≥5.6	24	14	
Tumor necrosis			
Minimal	23	6	0.43
Moderate to severe	32	14	

* Fisher's Exact test was used.

† Pathologic stage was used.

cause of death was either primary tumor recurrence ($n = 18$) or other causes ($n = 14$) including infection. Thus, the cancer-related death rate was 24%. The relatively high overall death rate reflects the high rate of comorbidity among veteran populations seen at Veteran's Affairs hospitals and an associated higher mortality rate in NSCLC (25).

To examine the importance of CA IX to survival, we performed multivariate analysis. To maximize the power of analysis, we used the three CA IX variables—MVD, Ki-67 index, and the extent of tumor necrosis—as continuous variables. Stage, race, and histology were used as binary variables (*i.e.*, stage I *versus* II, Caucasian *versus* African American, and squamous cell carcinoma *versus* non-squamous cell carcinoma). Gender was not included because all of the patients were male. The percentage of CA IX-positive tumor cells and Ki-67 index were strong independent prognostic indicators for disease-free survival ($P < 0.01$; Table 3). Pathologic stage II was also

significantly associated with shorter disease-free survival ($P = 0.04$). When patients were divided into two groups based on the median value of the percentage of CA IX-positive tumor cells, distinctly different disease-free survival times were found, as illustrated by Kaplan–Meier curves (Fig. 3A). In each stage, the percentage of CA IX-positive tumor cells was also significantly associated with shorter disease-free survival ($P < 0.01$, both Fig. 3B and C).

In contrast, CA IX score and the mean intensity of CA IX staining were not independent prognostic factors for disease-free survival when they were used as continuous variables in the multivariate analysis (data not shown). In addition, when all cases with non-zero staining of CA IX were considered as positive and the cases were dichotomized as a binary variable (positive or negative), there was no relationship with disease-free survival in univariate analysis ($P = 0.125$; data not shown).

In the multivariate analysis of overall survival, Ki-67 index and pathologic stage were significantly associated with worse overall survival ($P < 0.05$; Table 3). The percentage of CA IX-positive tumor cells also showed significance for overall survival ($P = 0.05$), indicating that a higher percentage of CA IX-positive tumor cells was associated with shorter disease-free survival (Table 3). Other variables including age, race, extent of necrosis, MVD, and histologic type were not significantly related to disease-free survival or overall survival ($P > 0.05$).

DISCUSSION

Several techniques have been used to measure tumor hypoxia, including oxygen microelectrodes (*e.g.*, Eppendorf pO₂ histogram) and nitromidazole derivatives (*e.g.*, EF5 and pimonidazole; refs. 4, 17, 26). However, the oxygen microelectrode measurement is an invasive procedure, and nitromidazole derivatives must be administered before obtaining the tumor sample. Thus, both methods require prospectively designed studies to evaluate the role of hypoxia in treatment outcome. Alternative methods of assessing hypoxia are required for application to archival samples and routine clinical practice.

Assessment of tumor hypoxia with endogenous hypoxia markers is an attractive alternative to the aforementioned methods. CA IX is one of several endogenous hypoxia markers that have been investigated. There is *in vitro* evidence that this protein is up-regulated under hypoxic conditions. For example,

Table 3 Cox proportional hazards regression model analysis of disease-free survival and overall survival

Variables	Disease-free survival			Overall survival		
	Coefficient	SEM *	P value	Coefficient	SEM *	P value
% of CA IX-positive cells †	0.267	0.083	<0.01	0.112	0.058	0.05
Ki 67 index (%) †	0.057	0.014	<0.01	0.028	0.010	<0.01
Stage II ‡	1.305	0.649	0.04	1.163	0.482	0.02
% of tumor necrosis †	−0.035	0.028	0.20	−0.022	0.020	0.28
Microvessel density †	−0.010	0.038	0.78	−0.005	0.028	0.84
Age *	0.002	0.034	0.95	0.003	0.023	0.87
Squamous cell carcinoma‡	0.441	0.545	0.41	0.561	0.432	0.19
African American ‡	−0.030	0.743	0.98	−0.153	0.547	0.78

* SEM of the coefficient.

† These variables were used as continuous variables with units of percent.

‡ Binary variables were used: stage I *versus* II, squamous cell carcinoma *versus* non-squamous cell carcinoma, Caucasian *versus* African American

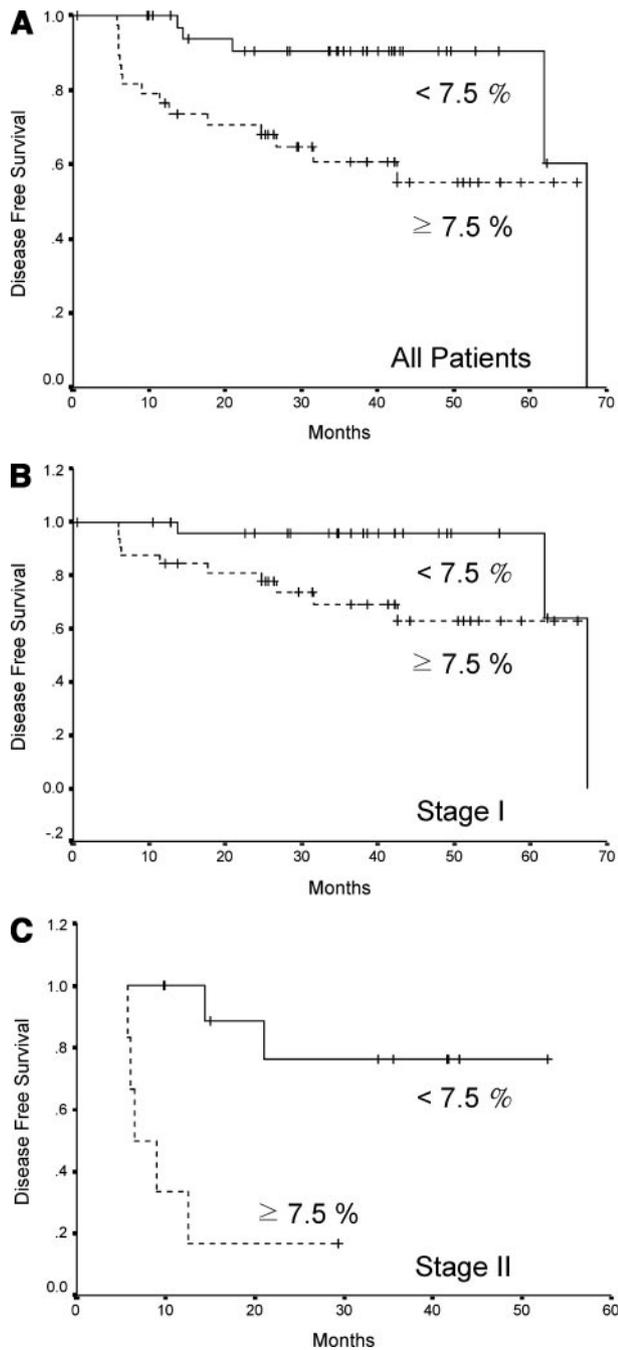


Fig. 3 Disease-free survival by percentage of CA IX-positive tumor cells. The median value of 7.5% was used as a cutoff value for grouping. In **A**, among all 75 cases, percentage of CA IX-positive cells is associated with shorter disease-free survival ($P < 0.01$). In **B** and **C**, high percentage of CA IX-positive cells is related with shorter disease-free survival in stage I and II ($P = 0.029$ and 0.003 , respectively). Curves were constructed with the method of Kaplan–Meier and compared by the Log-rank Test. Tick marks, censored data.

studies with various human cancer cell lines showed elevated levels of CA IX gene expression in response to hypoxia (27, 28). CA IX protein expression has been particularly strong when pO_2 levels are < 20 mmHg (11, 29). On the molecular level, the

promoter region of CA IX contains a hypoxia response element that is the binding site of the transcription factor, HIF-1 (30). The α subunit of HIF-1 is stabilized by hypoxia because, under normoxic conditions, HIF-1 α is rapidly degraded by proteasomes after being targeted for ubiquitination (6, 7).

Antibodies binding CA IX have facilitated its evaluation in biopsied tissue samples by immunohistochemistry. Initially, CA IX was reported to be expressed around necrotic areas in human tumor tissues, suggesting a relationship with hypoxia (8). A more definitive proof that it marks hypoxic regions in tumor tissues came from studies that correlated CA IX expression with the exogenous hypoxia marker, pimonidazole (17). However, there is controversy about how well CA IX identifies hypoxic regions. For example, the correlations were weak, and areas of mismatch between pimonidazole and CA IX were also found in the above-mentioned study. Furthermore, a lack of correlation was also found between oxygen microelectrode measurements and CA IX expression in carcinoma of uterine cervix (17, 18). Thus, the utility of CA IX as a hypoxia marker is still not established and should be thoroughly examined.

In our present study, CA IX expression was clearly related to the extent of necrosis, supporting its putative relation with hypoxia as reported previously (8). CA IX was predominantly expressed in areas adjacent to necrosis, and this perinecrotic expression is consistent with findings from other studies (19, 20). CA IX was more frequently expressed in squamous cell carcinoma than was adenocarcinoma. This is consistent with the fact that squamous cell carcinomas are more necrotic than other types of NSCLC (29). In addition, there was a weak but statistically significant inverse correlation between CA IX and MVD in our study, suggesting poor neovascularization is related to hypoxia-induced CA IX expression. Although high vascularization has been regarded as a predictor for aggressive behavior of tumors, the relationship between CA IX and MVD remains controversial. A direct relationship between CA IX and MVD was reported in one study of head and neck cancer (31) whereas an inverse relationship, similar to that found in our study, has been reported in another study of head and neck cancer (15). This discrepancy may be the result of heterogeneity of vascularization and various abnormalities of tumor vasculature (32, 33), or it may be the result of differences in the methods of evaluation.

To evaluate the prognostic significance of immunohistochemically detected CA IX expression in tumors, various indicators of CA IX staining have been used in previous studies: percentage of CA IX-positive tumor cells, positive or negative CA IX expression, and CA IX score (18–20, 34–36). However, the impact on survival varied across studies depending on the CA IX variables or tissue type. Direct comparison of the three variables in the same study population had not been performed. Thus, all three CA IX variables were compared in this study, which found that the percentage of CA IX-positive tumor cells was most strongly correlated with postoperative recurrence, disease-free survival, and overall survival. Thus, the percentage of tumor cells staining for CA IX may provide more information about tumor recurrence and prognosis of patients than grading the staining intensity. CA IX score, the product of staining intensity and percentage positive cells, was less informative than percentage itself. Therefore, measuring percentage of positively

stained cells appears to be the most informative assessment of CA IX expression.

Prognostic information can be maximized when variables are used as continuous variables instead of being reduced to a binary variable separated at a cut-point (37). Thus, we used continuous variables for multivariate analysis, and the percentage of CA IX-positive tumor cells was significantly associated with disease-free survival and overall survival in multivariate analysis. Together with pathologic stage and the percentage of CA IX-positive tumor cells, Ki-67 index was also significantly related with shorter disease-free survival and overall survival. Although prognostic significance of Ki-67 index has been reported in a variety of tumor types, including NSCLC, these studies used Ki-67 index as a cut-point–derived binary variable (38, 39). To our knowledge, this is the first study to demonstrate prognostic significance of CA IX and Ki-67 index when used as continuous variables in multivariate survival analysis. Prognostic significance of CA IX and Ki-67 index in our present study suggests a worse prognosis for more rapidly proliferating and more hypoxic early-stage NSCLCs. Recently, proteomic analysis identified another hypoxia-induced protein, the glycolytic enzyme phosphoglycerate kinase 1 (PGK1), as a poor prognostic predictor in lung adenocarcinoma, consistent with our findings (40).

It has been suggested that tumor cell proliferation-induced oxygen consumption might be a more important influence on the development of tumor hypoxia than reduced oxygen supply (41). Thus, as tumor cells proliferate, their microenvironment may become hypoxic. Although cells under prolonged hypoxic conditions are thought to proliferate slowly or die, the continuous proliferation of tumor cells in the hypoxic tumor compartment and the correlation between fast tumor cell doubling time and low tumor oxygenation have been previously reported (23, 42, 43). These findings suggested a possible interaction between cell proliferation and development of hypoxia. In the current study, the colocalization analysis demonstrated a significantly higher Ki-67 index in tumor areas that showed relatively high

CAIX expression. These findings are consistent with the previous report showing clear regional match between Ki-67 and CA IX in colorectal tumors (44). Ki-67 has been shown to present only in S, G₂, and M phases of the cell cycle as well as in the proliferation-associated part of G₁, but not in G₀ (45). Thus, when tumor cells are positively stained by Ki-67 in the area of CA IX expression, the presence of proliferating cells, not quiescent cells under hypoxia, are indicated.

The positive relationship between CAIX and Ki67 is somewhat surprising, as one would typically expect hypoxic cells to be nutrient deprived and therefore more likely to be quiescent. The findings of this study suggest two somewhat interrelated alternative mechanisms. First, it is possible that increased tumor cell proliferation increases oxygen consumption thereby leading to hypoxia and increased CAIX expression. Second, the expression of CA IX in hypoxic regions may help continued tumor cell proliferation by maintaining a pH adequate for cell survival under hypoxia. It has been reported that the oxygen consumption rate of proliferating cells is three to five times higher than that of quiescent cells (46). Proliferation of cells within a hypoxic environment will, by necessity, lead to increased acid production as a result of a switch from aerobic to anaerobic metabolism. CA IX, which is induced by hypoxia, produces bicarbonates via hydration of carbon dioxide into carbonic acid. Bicarbonates are exchanged for intracellular chloride to maintain the intracellular pH of tumor cells under hypoxia, and this allows tumors to acclimate to the acidic microenvironment, which is associated with increased anaerobic glycolysis (10, 11). Thus, the expression of CA IX may assist in maintaining intracellular pH at a level that will permit continued proliferation. Although the exact cause-and-effect relationships between hypoxia and cell proliferation could not be determined from the present study, hypoxia might provide survival advantage to proliferating tumor cells and promote clonal selection of viable hypoxic tumor cells with increased

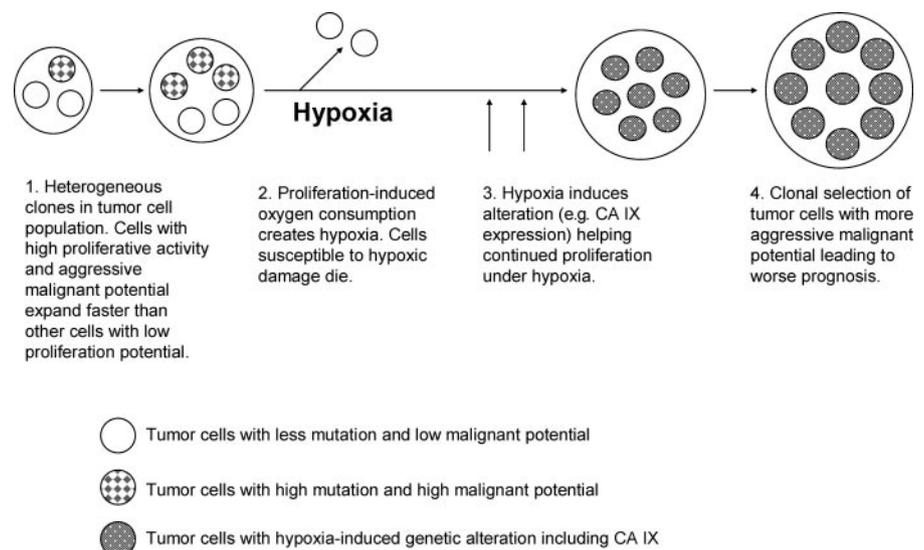


Fig. 4 Clonal selection of tumor cells via tumor hypoxia.

adaptation to hypoxia, driving the tumor toward a more aggressive phenotype (Fig. 4).

Although the staging system of the American Joint Committee on Cancer Staging is the accepted predictor of prognosis in lung cancer, the survival data of each stage have suggested the presence of heterogeneous populations containing patients at higher risk for recurrence than others within the same stage (21). Therefore, identifying patients at high risk for recurrence would allow the development of tailored treatment strategy to the particular patient. Considering the prognostic significance of CA IX and its relation with recurrence and cell proliferative index, CA IX expression status might be a selection tool for patients who require additional treatment before or after primary treatment.

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