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BIOLOGY CONTRIBUTION

EXPRESSION AND PROGNOSTIC SIGNIFICANCE OF A PANEL OF TISSUE HYPOXIA MARKERS IN HEAD-AND-NECK SQUAMOUS CELL CARCINOMAS

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Purpose: **To investigate the expression pattern of hypoxia-induced proteins identified as being involved in malignant progression of head-and-neck squamous cell carcinoma (HNSCC) and to determine their relationship to tumor pO_2 and prognosis.**

Methods and Materials: **We performed immunohistochemical staining of hypoxia-induced proteins (carbonic anhydrase IX [CA IX], BNIP3L, connective tissue growth factor, osteopontin, ephrin A1, hypoxia inducible gene-2, dihydrofolate reductase, galectin-1, IκB kinase β, and lysyl oxidase) on tumor tissue arrays of 101 HNSCC patients with pretreatment pO_2 measurements. Analysis of variance and Fisher's exact tests were used to evaluate the relationship between marker expression, tumor pO_2, and CA IX staining. Cox proportional hazard model and log–rank tests were used to determine the relationship between markers and prognosis.**

Results: **Osteopontin expression correlated with tumor pO_2 (Eppendorf measurements) ($p = 0.04$). However, there was a strong correlation between lysyl oxidase, ephrin A1, and galectin-1 and CA IX staining. These markers also predicted for cancer-specific survival and overall survival on univariate analysis. A hypoxia score of 0–5 was assigned to each patient, on the basis of the presence of strong staining for these markers, whereby a higher score signifies increased marker expression. On multivariate analysis, increasing hypoxia score was an independent prognostic factor for cancer-specific survival ($p = 0.015$) and was borderline significant for overall survival ($p = 0.057$) when adjusted for other independent predictors of outcomes (hemoglobin and age).**

Conclusions: **We identified a panel of hypoxia-related tissue markers that correlates with treatment outcomes in HNSCC. Validation of these markers will be needed to determine their utility in identifying patients for hypoxia-targeted therapy.** **© 2007 Elsevier Inc.**

Hypoxia, Marker panel, Head-and-neck cancer, Prognosis.

INTRODUCTION

Tumor hypoxia is a common phenomenon in solid tumors and has been shown to adversely affect treatment outcomes in patients with head-and-neck squamous cell carcinoma (HNSCC) treated with conventional therapy (1–6). There has been a strong interest in targeting hypoxia over the years. However, the clinical effort to exploit hypoxia has been impeded by the failure to accurately identify patients who would most benefit from hypoxia-targeted therapy. The negative results of several clinical trials can be attributed to patient heterogeneity and the fact that many studies were underpowered because not all enrolled patients would benefit from such therapy (7, 8).

A commonly accepted approach for assessing hypoxia has been by direct tumor pO_2 measurement with a polarographic electrode (Eppendorf electrode; Sigma Eppendorf pO_2 Histograph, Hamburg, Germany) (1–5). However, this is an invasive method in that it requires the placement of an electrode needle directly into the tumor for measurements. Other drawbacks of this approach include the high cost of the equipment, tumor inaccessibility, high interobserver variability, failure to distinguish necrosis from hypoxia, and the lack of spatial information on hypoxia (9, 10). Another method for assessing tumor

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hypoxia is through the administration of an injectable hypoxia marker, such as pimonidazole (1-(2-nitro-1-imidazolyl)-3-N-piperidino-2-propanolol) (11), and EF5 (nitroimidazole[2-(2-nitro-iH-imidazol-1-yl)-N-(2,2,3,3,3-pentaflouropropyl) acetamide) (9, 10, 12, 13). These compounds form stable adducts with intracellular macromolecules, and this binding is proportionally inhibited as a function of increasing oxygen concentration (14). Detection of these adducts with antibodies can provide information on the relative oxygenation of tissue at a cellular resolution. This approach is limited by the requirement for exogenous drug administration, additional biopsies for staining, and quantification of staining. Hypoxia imaging using specialized positron emission tomography or single photon emission computed tomography tracers is another promising approach. However, these technologies require expensive dedicated equipment for both imaging and tracer generation (9, 15).

Therefore, assessing hypoxia using endogenous molecular markers, which are proteins that are induced by hypoxic exposure, would be a cost-effective approach that could be used by oncologists worldwide. At present the most widely investigated proteins in tumor tissues are hypoxia inducible factor-1 (HIF-1) and carbonic anhydrase IX (CA IX) (16–18). Another advantage of endogenous molecular markers is that levels of these proteins can be assessed on archival materials, thereby allowing rapid correlation with treatment outcomes. In addition, molecular markers require neither injection of foreign material nor any additional invasive procedure beyond that of a tumor biopsy at diagnosis. However, a significant concern regarding this approach is that these proteins can be regulated by factors other than hypoxia. For example, HIF-1α expression can be influenced by several nonhypoxic stimuli, including nitric oxide, cytokines (interleukin-1β and tumor necrosis factor α), trophic stimuli (serum, insulin, insulin-like growth factors), and oncogenes (e.g., p53, Vsrc, PTEN) (19–22). Therefore, the inclusion of several endogenous markers could improve the specificity of such an approach to identifying patients with hypoxic tumors. To test this hypothesis, we performed a global study using gene array and proteomic analyses in HNSCC cell lines to identify novel hypoxia-regulated proteins that could be used as potential endogenous hypoxia markers (23–25). After confirming their hypoxic inducibility using conventional approaches, such as Northern, Western, and quantitative real-time polymerase chain reaction (PCR) analyses, we investigated their utility to predict outcomes by staining a tumor tissue array from HNSCC patients with known outcomes and tumor pO_2. These studies resulted in a panel of four hypoxia markers that can be used to predict treatment outcomes in terms of cancer-specific and overall survival.

METHODS AND MATERIALS

Cell culture, hypoxia treatment, gene array and proteomic analysis, Northern and immunoblot confirmation

These studies have been previously described in detail in references 23–26.

Tissue array generation and immunohistochemical staining

The tissue microarray was constructed as previously described (27). In addition to HNSCC, the array also included cores of skin, placenta, and benign lymph nodes as controls. Each sample was present in the microarray as duplicate cores.

Immunoperoxidase stains were performed on 4-μm-thick sections of the tissue microarray. Osteopontin (OPN) and galectin-1 (GAL-1) stains were also performed on 4-μm-thick whole sections of tumor. The following antibodies, dilutions, and incubation times were used: CA IX (1:50 dilution, 30 min at room temperature [RT], M75 mouse monoclonal (28)), GAL-1 (1:250, 30 min at RT, mouse monoclonal; Novocastra), ephrin A1 (1:100, 30 min at RT, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), IκB kinase β (IKKβ) (1:1000, 30 min at RT, mouse monoclonal; Santa Cruz Biotechnology), lysyl oxidase (LOX) (1:20; 30 min at RT, polyclonal rabbit antibody raised against EDTSCDYGYHRRFA; Open Biosystems, Novocastra, Newcastle upon Tyne, UK) (26), BNIP3L (1:150, 30 min at RT, antipeptide rabbit polyclonal antisera; Open Biosystems, Novocastra, Newcastle upon Tyne, UK) (29), OPN (1:50, 18 h at 4°C, rabbit monoclonal; Lab Vision Corporation, Fremont, CA), dihydrofolate reductase (DHFR) (1:500, 30 min at RT, goat polyclonal; Santa Cruz Biotechnology), hypoxia inducible gene-2 (HIG2) (1:200, 30 min at RT, antipeptide rabbit polyclonal antisera), and connective tissue growth factor (CTGF) (1:400, 30 min at RT, rabbit polyclonal antibody directed against mouse CCN2/Fisp12, which cross-reacts with human CTGF; generously provided by Dr. L. Lau) (30). The slides were deparaffinized in xylene, then rehydrated before 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 phosphate-buffered saline wash, the slides were incubated with the secondary antibody and 3,3′-diaminobenzidine (DAKO Envision Kit; DAKO, Glostrup, Denmark). After a hematoxylin counterstain, the slides were coverslipped.

All slides were reviewed by a pathologist who was blinded to treatment outcomes and scored as follows: negative = 0, equivocal or uninterpretable = 1, weakly positive = 2, strongly positive = 3. The CA IX staining pattern was scored separately for cytoplasmic and membrane staining to account for the fact that these proteins are localized to multiple intracellular compartments.

Patients

Criteria for patient participation in the study include (*1*) newly diagnosed HNSCC, (*2*) presence of involved lymph node or tumor that was accessible for microelectrode measurement, and (*3*) willingness to sign an informed consent approved by the Stanford institutional review board. The patient study group consisted of 101 adults. The staging evaluation for all patients included history and physical examination, panendoscopy, chest radiographs, and laboratory tests. All patients had head-and-neck computed tomography or magnetic resonant imaging studies. All were staged according to the 1988 American Joint Committee on Cancer staging system (31). All patients were treated with either chemoradiotherapy or surgery plus radiotherapy. Patients were initially followed monthly after primary treatment and then at longer intervals. All first relapses were documented by tissue biopsy. Table 1 shows the patient pretreatment and treatment characteristics.

Tumor pO_2 measurement

All measurements were performed using a computerized histograph (Sigma Eppendorf PO_2 Histograph, Hamburg, Germany) as previously described (32). The measurements were presented in

Table 1. Patient and treatment characteristics

Parameter	No. of patients
Age (y) (median: 58)	
<60	55
≥60	46
Gender	
Male	84
Female	17
Site	
Oropharynx	62
Larynx	8
Oral cavity	13
Hypopharynx	12
Other	6
T stage	
0–2	48
3–4	53
N stage	
0–1	15
2	66 (2A = 9, 2B = 34, 2C = 23)
3	20
Stage	
III	10
IV	91
Hemoglobin (g/dL)	
≤12	17
>12	79
Unknown	5
Median tumor pO_2 (mm Hg) (median: 11)	
≤10	43
>10	52
Not evaluable	6
Treatment	
Chemoradiation	80
Surgery ± radiation	21

the form of histograms along with the calculation of a median pO_2 and the hypoxic fraction <5 mm Hg (HF5) for each site. In all patients the median tumor pO_2 was consistently lower than that of normal subcutaneous tissues.

Statistical analysis

Statistical analysis was performed using R version 2.2.0 and the Hmisc and Design packages contributed by Frank Harrell. Analyzed outcomes included head-and-neck cancer-specific survival (CSS, with death from HNSCC counting as an event and other deaths as censoring times) and overall survival (OS). They were computed using the Kaplan-Meier product-limit method, and the significance of individual predictive variables was tested with the log–rank test. Correlations among predicting variables were assessed by the Pearson coefficient, odds ratios, and other graphic means. Statistical significance was assumed at $p < 0.05$, and all tests were two sided. Analysis of variance (ANOVA) was used to correlate marker staining with median tumor pO_2 and HF5. Fisher's exact test was used to evaluate the relationship between CA IX staining and that of other hypoxia markers. A Cox proportional hazard model was conducted to evaluate the independent prognostic significance of hypoxia score in relation to clinical parameters that were found to be significant on univariate analysis for evaluated outcomes (CSS and OS). To avoid prejudging the form of the relationship between the hypoxia score and CSS/OS, we used overall tests of equality of survival curves (5 degrees of freedom, comparing 6 curves) rather than assuming a monotonic or log-linear effect on the hazard.

RESULTS

Identification of hypoxia markers in vitro

Table 2 shows the list of analyzed tissue markers, their putative function, their hypoxia-inducible form (messenger ribonucleic acid [mRNA] vs. protein), and their subcellular distribution. All markers had at least twofold or greater induction by hypoxia at either the mRNA or protein level. Validation was performed for all markers using Northern blot, real-time quantitative PCR, or immunoblot studies. Detailed analyses of several markers in cell lines, including BNIP3L, LOX, OPN, DHFR, IKKβ, HIG2, GAL-1, and ephrin A1 have been previously published (23–26).

Correlation between hypoxia markers and tumor oxygenation

Table 3 shows the staining distribution for individual hypoxia markers in patients with evaluable hypoxia marker staining and tumor pO_2. Carbonic anhydrase IX, which has been shown to exist in more than one subcellular compartment, was scored separately for each subcellular location. Figure 1 shows representative marker staining for CA IX (cytoplasmic and membrane), LOX, GAL-1, and ephrin A1. Hematoxylin and eosin staining was performed on consecutive sections to confirm the presence of tumors in each core. As shown in Table 3, strong tumor staining was noted for a large percentage of patients for OPN and in a very small percentage of patients for DHFR and IKKβ. Only OPN tumor staining showed a statistical correlation to median tumor pO_2 by ANOVA ($p = 0.04$; Table 3, Fig. 2). In addition, positive IKKβ staining (either weak or strong) was significantly associated with a higher hypoxic fraction (HF5, $p = 0.03$). There was a trend for decreasing tumor pO_2 and increasing HF5 with strong GAL-1 staining, though the difference did not reach statistical significance.

Table 4 shows the correlation of marker staining with CA IX staining. For this analysis, CA IX staining was considered positive when the tumor had either strong membrane and/or cytoplasmic staining. It was considered as weak or negative if there was no strong staining in either compartment. Markers that showed significant correlation to CA IX staining were BNIP3L ($p = 0.01$), LOX ($p = 0.0001$), CTGF ($p = 0.001$), ephrin A1 ($p < 0.0001$), and GAL-1 ($p = 0.005$).

Correlation between hypoxia markers, clinical parameters, and treatment outcomes

At a median follow-up of 58 months for living patients, 48 have died (36 from progressive or recurrent cancer and 12 from intercurrent illnesses). The 5-year CSS and OS rates for the entire group were 64% and 53%, respectively. Forty patients have relapsed. The site of the first relapse was local alone in 7, nodal alone in 9, locoregional alone in 12, nodal and distant alone in 2, and distant alone in 10 patients.

We evaluated tissue hypoxia markers for their relationship to CSS and OS. Univariate analyses revealed that similar results were obtained when each marker was evaluated as a trichotomous (negative vs. weak vs. strong staining) or as

Table 2. Characteristics of analyzed endogenous tissue hypoxia markers

Marker	Putative function	Major hypoxia-inducible form	Subcellular localization	Strongly positive staining (%)
BNIP-3L	Mitochondrial	mRNA and protein	Cytoplasmic	60
LOX	Collagen matrix stabilization, metastasis	mRNA and protein	Secreted	60
OPN	Transformation, metastasis	mRNA, secreted protein	Secreted	83
DHFR	dTMP biosynthesis, response to chemotherapy	Protein	Cytoplasmic	12
IKKβ	NF$\kappa\beta$ activation	Protein	Cytoplasmic	5
HIG-2	Wnt signaling, autocrine growth factor	mRNA and protein	Cytoplasmic	31
Gal-1	Carbohydrate binding protein, suppression of immune surveillance	Secreted protein	Secreted	56
Ephrin A1	Angiogenesis	mRNA and protein	Secreted	28
CTGF	Connective tissue organization, adhesion	mRNA and protein	Secreted	53
CA IX (Carbonic Anhydrase IX)	pH Regulation	mRNA & protein	Cytoplasmic/ membrane	39 (cytoplasmic) 29 (membrane)

Abbreviations: BNIP-3L = BCL2adenovirus E1B 19kD-interacting protein 3 long; LOX = lysyl oxidase; OPN = osteopontin; DHFR = dihydrofolate reductase; dTMP = deoxythymidine monophosphate; IKKβ = IκB kinase β; NF$\kappa\beta$ = nuclear factor $\kappa\beta$; HIG-2 = hypoxia-inducible gene-2; Gal-1 = galectin-1; CTGF = connective tissue growth factor; CA IX = carbonic anhydrase IX.

a dichotomous variable (negative–weak vs. strong staining). Therefore, we classified all markers as a dichotomous variable (negative–weak vs. strong) for ease of analysis. Tissue markers that achieved a statistically significant difference for both CSS and OS on univariate analyses were LOX, CA IX membrane, CA IX cytoplasmic, ephrin A1, and GAL-1 (Table 5). Because CA IX cytoplasmic and membrane staining were unrelated to each other according to

Table 3. Relationship between tumor marker staining and tumor oxygenation as measured by the Eppendorf Histograph

Marker	Staining intensity	*n* (%)*	Median pO_2 (mm Hg)	*p* (ANOVA)	HF5 (%)	*p* (ANOVA)
BNIP-3L (*n* = 87)	Negative	16 (19)	13.4	0.90	33.5	0.21
	Weak	21 (24)	15.1		19.5	
	Strong	50 (57)	15.0		33.7	
LOX (*n* = 89)	Negative	23 (26)	14.3	0.46	31.5	0.69
	Weak	14 (16)	18.2		24.7	
	Strong	52 (58)	13.6		33.1	
OPN (*n* = 84)	Negative	4 (5)	28.7	0.04†	0.8	0.14
	Weak	10 (12)	15.1		36.3	
	Strong	70 (83)	13.2		32.4	
DHFR (*n* = 88)	Negative	16 (19)	17.4	0.31	22.2	0.18
	Weak	62 (70)	13.5		34.2	
	Strong	10 (11)	18.7		18.3	
IKKβ (*n* = 87)	Negative	14 (16)	19.9	0.19	10.9	0.03†
	Weak	68 (78)	13.4		25.1	
	Strong	5 (6)	16.7		26.4	
HIG-2 (*n* = 90)	Negative	54 (60)	12.8	0.21	35.1	0.41
	Weak	10 (11)	17.5		26.2	
	Strong	26 (29)	17.5		25.7	
Gal-1 (*n* = 94)	Negative	14 (15)	13.2	0.15	29.6	0.07
	Weak	29 (31)	18.0		32.7	
	Strong	51 (54)	12.6		33.4	
Ephrin A1 (*n* = 94)	Negative	16 (17)	17.0	0.22	28.7	0.36
	Weak	53 (56)	15.4		28.9	
	Strong	25 (27)	11.0		39.6	
CA IX membrane (*n* = 94)	Negative	63 (67)	14.4	0.47	33.6	0.73
	Weak	2 (2)	17.9		26.1	
	Strong	29 (31)	16.6		28.2	
CA IX cytoplasmic (*n* = 94)	Negative	8 (9)	15.2	0.70	29.2	0.94
	Weak	51 (54)	13.5		31.3	
	Strong	35 (37)	15.7		33.2	

Abbreviation: ANOVA = analysis of variance; HF5 = hypoxic fraction <5 mm Hg. Other abbreviations as in Table 2.
* Only patients with interpretable staining patterns for each marker and evaluable pO_2 measurements were included in this table.
† $p < 0.05$.

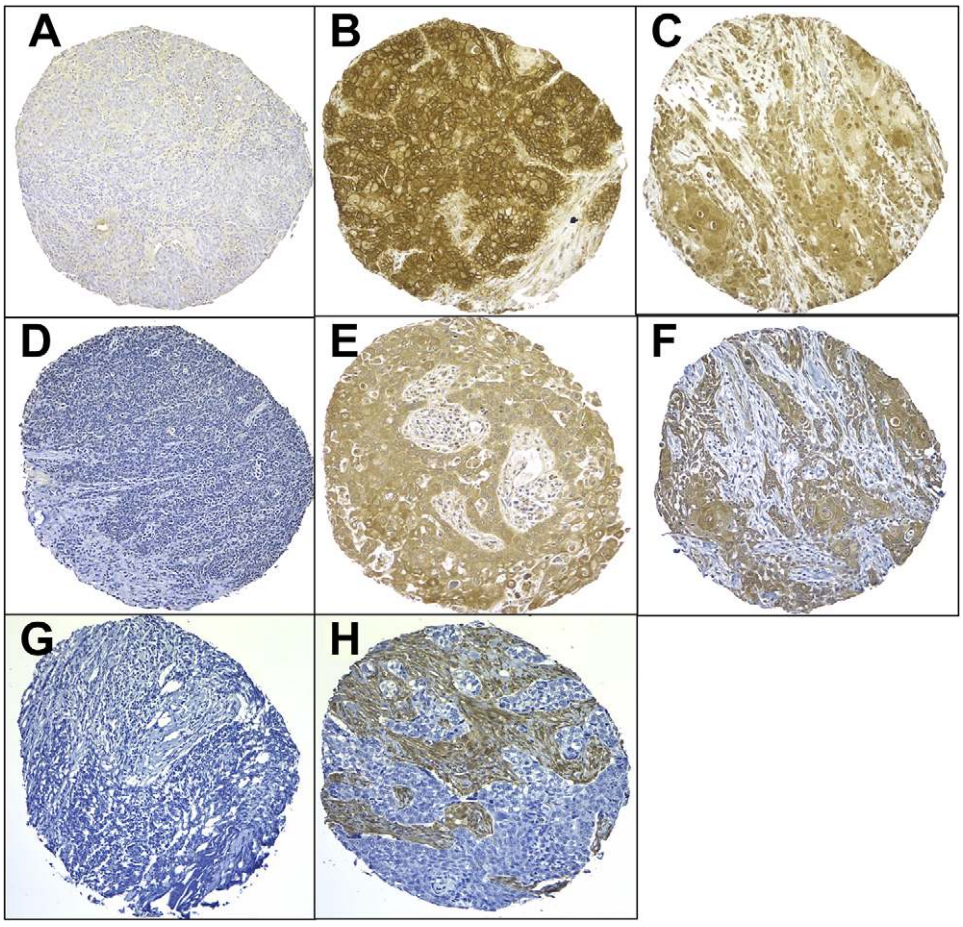

Fig. 1. Representative staining of different tissue hypoxia markers on tumor cores from the tissue microarray. (A) Negative staining for carbonic anhydrase IX (CA IX); (B) strong CA IX membrane staining; (C) strong CA IX cytoplasmic staining; (D) negative staining for lysyl oxidase (LOX); (E) strong LOX staining; (F) strong ephrin A1 staining; (G) negative staining for galectin-1; (H) strong galectin-1 staining.

odds ratio analysis, we considered them as two separate markers. We then used these five markers together to assign each patient a tissue hypoxia marker score equal to the number of markers staining strongly (0–5). A small number of patients had equivocal staining for one or more of the markers, resulting in some underestimation of the score, which was based on the observed markers. Repeating the analysis in subsets defined by the patterns of missing data did not substantially change the results.

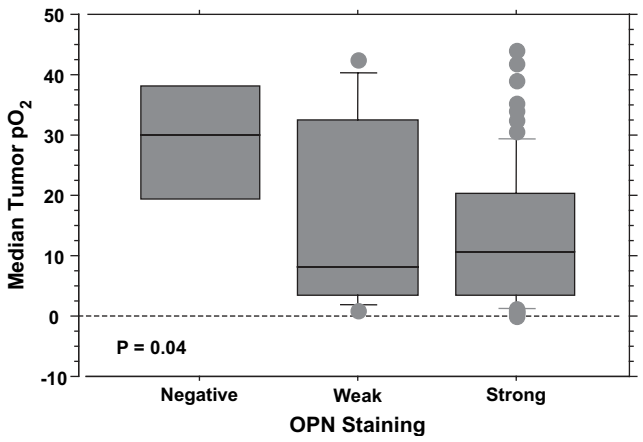

Fig. 2. Box plots showing correlation between osteopontin (OPN) expression and median tumor pO_2; $p = 0.04$ by analysis of variance.

We first evaluated for any possible association between hypoxia score and any clinical parameters in Table 1. There was no significant association between age, gender, hemoglobin, T stage, N stage, or overall stage. There was a significant association between hypoxia score and primary tumor site, with more high scores observed for non-oropharyngeal compared with oropharyngeal sites (score 4–5: 44% vs. 10%, $p = 0.005$, Kendall Rank correlation). Similarly, there was a significant association between hypoxia score and treatment, with more high scores in surgically treated compared with chemoradiotherapy-treated patients (score 4–5: 52% vs. 15%, $p = 0.0003$, Kendall Rank correlation). However, significantly more patients with non-oropharyngeal tumors were treated surgically than those with oropharyngeal tumors ($p = 0.05$, chi-square).

To avoid prejudging the form of the relationship between the hypoxia score and CSS/OS, we used overall tests of equality of survival curves (5 degrees of freedom, comparing 6 curves) rather than assuming a monotonic or log-linear effect on the hazard. Both CSS and OS depend significantly on

Table 4. Relationship between tumor hypoxia marker and CA IX staining

Marker	Staining intensity	No. (%) weak–negative CA IX	No. (%) strong CA IX	*p*
BNIP3L	Negative–weak	23 (25)	15 (16)	0.01*
	Strong	18 (19)	36 (39)	
LOX	Negative–weak	24 (27)	8 (9)	0.0001*
	Strong	19 (21)	38 (43)	
CTGF	Negative–weak	26 (30)	15 (17)	0.001*
	Strong	12 (14)	33 (38)	
OPN	Negative–weak	7 (8)	8 (9)	0.99
	Strong	34 (38)	40 (45)	
Ephrin A1	Negative–weak	44 (44)	28 (28)	< 0.0001*
	Strong	2 (2)	25 (25)	
HIG2	Negative–weak	31 (33)	34 (36)	0.27
	Strong	10 (11)	19 (20)	
GAL-1	Negative–weak	27 (27)	17 (17)	0.005*
	Strong	18 (18)	38 (38)	
DHFR	Negative–weak	37 (40)	45 (48)	0.75
	Strong	6 (7)	5 (5)	
IKKβ	Negative–weak	43 (46)	46 (49)	0.37
	Strong	1 (1)	4 (4)	

Abbreviations as in Table 2.
* $p < 0.05$.

the hypoxia score (the *p* values for the null hypothesis that all 6 curves are equal are <0.0001 for CSS and 0.003 for OS), and there is evidently a trend to lower CSS and OS with increasing score.

In addition to the above-named tissue hypoxia markers, other significant parameters for CSS and OS on univariate analyses were primary tumor site (favoring oropharyngeal sites), treatment (favoring chemoradiotherapy), and hemoglobin (favoring the highest tertile; Table 5). When adjusted for the tissue hypoxia marker score, tumor site and treatment were no longer significant; therefore only hemoglobin and hypoxia marker score were included in the multivariate model for CSS and OS. Age was also included in the final model because it has been shown to be a consistent prognostic factor for survival in head-and-neck cancer patients. Table 6 shows the result of the multivariate analyses for CSS and OS. Hypoxia score was highly significant for CSS and of borderline significance for OS. Figure 3 shows CSS (Fig. 3a) and OS (Fig. 3b) according to hypoxia marker score, adjusted for age and hemoglobin level. Figure 4 shows the relative hazard ratio for age, hemoglobin, and hypoxia score for CSS on the multivariate model, illustrating that the hypoxia scores from 1 to 3 approximately double the cancer-specific hazard over the baseline score 0, and that scores of 4 or 5 at least redouble the hazard. Competing risks survival regression shows a highly significant effect of hypoxia score on CSS ($z = 3.02$, $p = 0.002$), adjusting for both age and hemoglobin, with no significant effect on non-cancer mortality ($z = -1.67$, $p = 0.094$).

DISCUSSION

Tumor oxygenation with solid tumors is relatively heterogeneous and highly dependent on both oxygen consumption and oxygen delivery. These opposing factors result in a dynamic equilibrium within tumors that ultimately determines the state of ''global tumor hypoxia.'' Assessing the staining pattern from a single biopsy can only yield a snapshot of

Table 5. Significant parameters for head-and-neck cancer-specific and overall survival on univariate analyses

		CSS		OS	
Parameter	Favored	Chi-square	*p*	Chi-square	*p*
LOX	Negative–weak	6.3	0.012	4.5	0.033
CA IX membrane	Negative–weak	4.6	0.031	4.1	0.044
CA IX cytoplasmic	Negative–weak	6.6	0.011	4.5	0.030
Ephrin A1	Negative–weak	7.5	0.006	6.5	0.010
Galectin-1	Negative–weak	6.5	0.010	7.1	0.008
CTGF	Negative–weak	5.2	0.022	1.9	0.164
Tumor site (OP vs. other)	OP	6.5	0.010		
Treatment	Chemoradiation	11.8	0.006	5.8	0.016
Hemoglobin*	Continuous*	7.0	0.030	9.1	0.010

Abbreviations: CSS = cancer-specific survival; OS = overall survival; OP = oropharynx. Other abbreviations as in Table 2.
* For continuous variables, the HR and its CI compare the highest and lowest quartiles.

Table 6. Results of multivariate analysis for head-and-neck cancer-specific and overall survival

	CSS			OS		
Parameter	Chi-square	HR (95% CI)	*p*	Chi-square	HR (95% CI)	*p*
Hypoxia score	14.03		0.015	10.71		0.057
1:0		1.85(9.44–7.77)			3.92 (1.05–14.63)	
2:0		1.42 (0.33–6.05)			3.20 (0.87–11.83)	
3:0		2.16 (0.48–9.78)			3.43 (0.85–13.85)	
4:0		4.45 (1.22–16.22)			4.78 (1.31–17.38)	
5:0		9.71 (2.10–44.97)			11.64 (2.51–54.08)	
Age*	2.08	1.35 (0.90–2.01)	0.149	5.25	1.55 (1.07–2.26)	0.022
Hemoglobin*	4.77	0.58 (0.36–0.95)	0.028	6.63	0.57 (0.38–0.88)	0.010

Abbreviations: HR = hazard ratio; CI = confidence interval; CSS = cancer-specific survival; OS = overall survival.
* For continuous variables, the HR and its CI compare the highest and lowest quartiles.

this dynamic process. Furthermore, the stability of various hypoxia-regulated proteins can also influence the local protein expression. Therefore, it is extremely unlikely that a single endogenous marker of hypoxia will reflect the true state of global hypoxia within tumors. To address these issues, we analyzed the expression pattern of a panel of hypoxic markers to characterize the extent of tumor hypoxia with high accuracy. First, we performed a comprehensive study to identify genes and proteins whose expressions are induced by hypoxia in head-and-neck cancer cell lines using genomic and proteomic approaches. The increased expressions of mRNA or proteins were validated using conventional methods, including immunoblots, Northern blots, and/or real-time quantitative PCR. Then we investigated the usefulness of using these proteins to predict for survival in HNSCC tumors by staining for their expression on a tissue microarray containing 101 tumor samples from HNSCC patients treated at our institution with known outcomes and tumor pO_2. We chose to focus on these proteins because they have been previously suggested to involve in malignant tumor progression.

The functions of several of these hypoxia markers have been previously described (24–26, 29). One new marker in the hypoxia score is ephrin A1, which was originally identified as a tumor necrosis factor α (TNF-α) inducible gene in human umbilical vein endothelial cell (33). It is highly expressed in the developing vasculature during embryogenesis

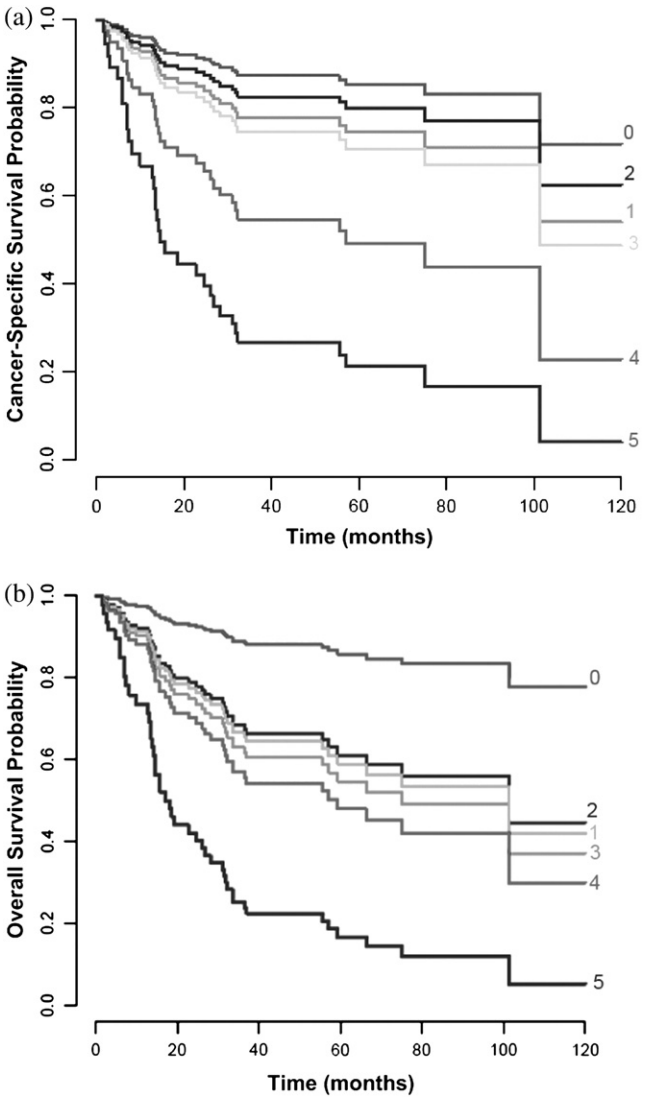

Fig. 3. (a) Head-and-neck cancer-specific survival by hypoxia marker score, adjusted for age and hemoglobin levels. (b) Overall survival by hypoxia marker score, adjusted for age and hemoglobin levels.

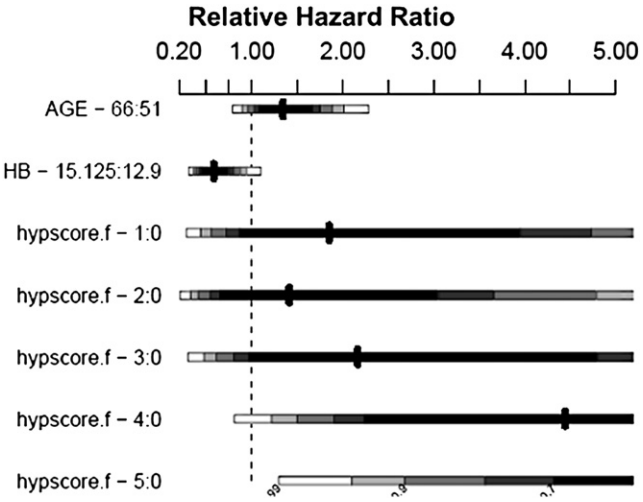

Fig. 4. Relative hazard ratio for age, hemoglobin (HB), and hypoxia marker score on a multivariate model for cancer-specific survival.

(34). It is involved in endothelial cell migration, capillary assembly, and angiogenesis, and its signaling is critical for normal blood vessel development (35, 36). Ephrin A1 has also been suggested to play a role in pathologic angiogenesis in adulthood because it is found to be highly expressed during tumor neovascularization (37). The interaction between ephrin A1 and its receptor seems to be essential for induction of maximal neovascularization by vascular endothelial growth factor (VEGF). In tumors, expression of ephrin A1 may be induced by various growth factors and cytokines, including TNF-α), lipopolysaccharides, interleukin-1β, and VEGF (38). In a mouse skin flap model of hypoxia, both ephrin A1 and its receptor were induced in the skin, and the induction seemed to be modulated by HIF-1α because small interfering RNA to HIF-1α could abrogate hypoxia induction of ephrin A1 (39). In a gene array analysis, ephrin type A receptor 1 was found to be positively correlated with increasing necrosis in human glioblastomas and was found to be localized to the perinecrotic areas on immunohistochemical staining (40). Our data confirmed hypoxia induction of ephrin A1 in head-and-neck cancers.

One significant finding was the lack of correlation between tumor pO_2 as assessed by the Eppendorf electrode with known makers of hypoxia, such as CA IX and BNIP3L. The only marker that had any significant correlation to tumor pO_2 was OPN, and GAL-1 was of borderline significance. The lack of correlation between these markers and tumor pO_2 can be explained by several reasons, including the heterogeneity in tumor pO_2 measurements over time (data were collected over a period of 10 years), influence of necrosis and pressure on tumor pO_2 measurement, and induction of these markers by factors or oncogenes other than hypoxia. However, there was a significant correlation between tumor staining of CA IX and of other hypoxia markers, including LOX, CTGF, ephrin A1, BNIP3, and GAL-1. All of these proteins, with the exception of GAL-1, seem to be regulated by HIF-1α at the mRNA levels, and this common regulation may explain the correlation of expression.

The gold standard for prognostic biomarkers is treatment outcome as measured by survival parameters, such as CSS and OS. We hypothesize that multiple markers may be better than a single marker in reflecting the tumor physiology and its microenvironment and in predicting survival. Precedence for this concept has been shown in the superiority of gene expression profiling over known clinical prognostic markers and individual biomarkers in identifying tumor subtypes and in predicting treatment outcomes in breast cancers and lymphomas (41–45). On the basis of univariate analysis, we developed a hypoxia score that incorporates for different markers and five different staining patterns. The hypoxia score was a strong predictor for CSS on multivariate analysis, accounting for tumor site, treatment, stage, age, and hemoglobin. There was a trend for increasing head-and-neck cancer-related death with increasing hypoxia marker score. At this point the sample size is too small to distinguish between a ''threshold'' effect and a smoothly increasing risk across the entire range of hypoxia scores. The decreased significance of hypoxia score on multivariate analysis for OS can be explained by the high number of non–head-and-neck cancer-related deaths in these patients. Because this is a cohort of older patients with mature follow-up, 1 of 4 deaths (12 of 48) occurred years after treatment, predominantly from either cardiopulmonary disease or secondary malignancies. This is corroborated by competing risk analysis, which showed increasing rate of head-and-neck cancer-specific mortality with hypoxia score, whereas there is no relationship between hypoxia score and non–cancer-related deaths.

In summary, we have generated a hypoxia marker score based on a group of hypoxia-induced genes and proteins that can be used to predict survival in HNSCC. Drawbacks from this study include the small sample size and patient and treatment heterogeneity. The results of this study will need to be validated in a larger cohort of homogeneously treated patients, which we hope to accomplish using tumor samples from patients who have completed treatment on the HeadSTART trial (15). In this large, Phase III, randomized study, 850 patients with locally advanced HNSCC were randomized to either cisplatin and radiation alone or to the same treatment plus tirapazamine, a hypoxic cell cytotoxin. The results obtained from tumor samples of the HeadSTART study will allow us to validate the prognostic significance of our hypoxia marker panel and to determine their usefulness in identifying patients who would benefit from hypoxia-targeting agents, such as tirapazamine.

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