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Research Article

Molecular Cancer Therapeutics

Serum Signature of Hypoxia-Regulated Factors Is Associated with Progression after Induction Therapy in Head and Neck Squamous Cell Cancer

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Abstract

Tumor hypoxia regulates many cytokines and angiogenic factors (CAF) and is associated with worse prognosis in head and neck squamous cell cancer (HNSCC). Serum CAF profiling may provide information regarding the biology of the host and tumor, prognosis, and response to therapy. We investigated 38 CAFs in HNSCC patients receiving induction therapy on a phase II trial of carboplatin, paclitaxel, and cetuximab. CAFs were measured by multiplex bead assay and enzyme-linked immunosorbent assay in 32 patients. Baseline and postinduction CAF levels were correlated with disease progression (PD) and human papilloma virus (HPV) status by Wilcoxon rank sum test. Baseline levels of eight hypoxia-regulated CAFs (the "high-risk signature" including vascular endothelial growth factor, interleukins 4 and 8, osteopontin, growth-related oncogene-α, eotaxin, granulocyte-colony stimulating factor, and stromal cell–derived factor-1α) were associated with subsequent PD. Elevation in ≥6 of 8 factors was strongly associated with shorter time to progression (P = 0.001) and was 73% specific and 100% sensitive for PD. Increasing growth-related oncogene-α from baseline to week 6 was also associated with PD. Progression-free and overall survival were shorter in patients with HPV-negative tumors (P = 0.012 and 0.046, respectively), but no individual CAF was associated with HPV status. However, among 14 HPV-negative patients, the high-risk CAF signature was seen in all 6 patients with PD, but only 2 of 14 without PD. In conclusion, serum CAF profiling, particularly in HPV-negative patients, may be useful for identifying those at highest risk for recurrence. *Mol Cancer Ther; 9(6); 1755–63. ©2010 AACR.*

Introduction

Annually, 47,500 people present with new HNSCC in the United States (1), and 30% to 40% of patients will relapse within 2 to 3 years following traditional therapy with radiation or chemoradiation (2). A significant subset of HNSCC tumors have hypoxic regions (3), which are an independent predictor of poor outcome after radiotherapy or combined treatments (4–6). The downstream effects of tumor hypoxia on hypoxia-inducible factor-1α (HIF1α) activation and angiogenesis may contribute to resistance to radiation and to the epidermal growth factor receptor (EGFR) inhibitor cetuximab (7, 8).

In vitro studies suggest that 1% to 1.5% of all genes are hypoxia regulated, many of which are part of signaling pathways that promote cancer proliferation, angiogenesis, and progression (4). Recently, Harris et al. identified a 99-metagene signature of tumor hypoxia that correlates with clinical outcome in HNSCC patients (9, 10). Because many cytokines and angiogenic factors (CAF) are hypoxia regulated, we hypothesized that a profile of serum CAFs would correlate with clinical outcome. Earlier studies have suggested a potential role for blood-based biomarkers in detecting HNSCC among high-risk patients (11–14) and as independent predictors of poor outcome in HNSCC (15, 16). More recently, Allen et al. observed that increasing levels in five NF-κB–modulated cytokines [interleukin (IL)-6 and IL-8, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), or growth-related oncogene-α (Gro-α)] were associated with shorter cause-specific survival (16). Because hypoxia can modulate secreted proteins through multiple pathways, we investigated a broad panel of 38 CAFs in patients receiving induction therapy for locally advanced

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HNSCC. In addition, because human papilloma virus (HPV)–positive tumors have better clinical outcomes and seem to have a distinct biology from HPV-negative tumors (17–19), we also investigated whether HPV status affects a patient's CAF profile.

Blood-based biomarkers are practical for monitoring during and after treatment. Furthermore, serum factors also reflect the contribution of the microenvironment and host immune response to the behavior of HNSCC, unlike techniques that directly assess only the tumor cells.

In this study, we used multiplex bead assay and enzyme-linked immunosorbent assay (ELISA) to perform an exploratory analysis of 38 CAFs in serum from patients treated on a phase II induction chemotherapy trial (20). We identified eight baseline CAFs that were individually associated with outcome. The association was even stronger when they were combined together into a "high-risk" signature. Although individual CAF levels were not associated with HPV status, elevations in high-risk CAFs were observed in HPV-negative patients with subsequent progressive disease (PD).

Materials and Methods

Induction chemotherapy study design and treatment outcome

Forty-seven previously untreated patients (33 male, 14 female) with advanced nodal disease ($T_{1–4}$, $N_{2b/c/3}$, M_0), Eastern Cooperative Oncology Group performance status 0 to 1, received six weekly cycles of neoadjuvant paclitaxel (135 mg/m^2), carboplatin (AUC 2), and cetuximab (400 mg/m^2, week 1; 250 mg/m^2, weeks 2–6) as part of a phase II clinical trial at M.D. Anderson Cancer Center (protocol 2003:0919; ref. 20). A majority of patients enrolled had oropharyngeal primary tumors (n = 42). Local therapy following chemotherapy was risk based, with baseline $T_{1–2}$ tumors receiving radiation alone (or surgery in one patient with oral primary) and $T_{3–4}$ tumors receiving concurrent chemoradiation. Neck dissection was done in nine patients with residual metastasis after locoregional treatment. Written informed consent was obtained from each patient after approval of the study by The University of Texas M.D. Anderson Cancer Center Institutional Review Board.

Following induction therapy, but before local therapy, patients were evaluated for clinical and radiographic response in the primary tumor and regional lymph nodes. Nine (19%) of 47 patients had an overall clinicoradiographic complete response, 36 (77%) had a partial response, and 2 (4%) had stable disease. Complete response was more common in never smokers (38.9%) versus former (12.5%) or current smokers (0%).

Patients were followed every 3 months for routine surveillance. At a minimum follow-up of 2 years (median, 33 months), six patients have had PD (two local; one local and distant; two regional and distant; and one distant). Four of these patients died of HNSCC; two are without evidence of disease following salvage surgery. The remaining 41 patients (87%) are alive without evidence of disease.

Serum collection and preparation for CAF analysis

Baseline serum was collected within 1 week of starting induction therapy. Serum was also collected in 26 of 32 patients at the end of induction chemotherapy but before definitive treatment. Peripheral blood samples were collected by routine venipuncture technique and allowed to coagulate for 30 to 60 minutes. Samples were centrifuged for 10 to 15 minutes at 1,000 × g to separate serum, which was then frozen and stored at −70°C to −80°C.

Multiplex bead assay and ELISA

Thirty-eight CAFs were measured by multiplex bead assay (36 factors) or ELISA (2 factors). These included hypoxia-induced factors, chemokines, interleukins, angiogenic factors, apoptosis mediators, and hematopoietic growth factors (Table 1). Multiplex bead assays were done with BioSource Multiplex Assays for Luminex (Invitrogen) in a 96-well format according to the BioSource protocol. Multiplex bead assay is a high-throughput technology that uses antibody-coupled

Table 1. Cytokines and angiogenic factors in serum biomarker analysis

βNGF	IL-18	MIF
CTACK	IL-1RA	MIG
Eotaxin	IL-2	MIP-1β
G-CSF	IL-2RA	Osteopontin
GRO-α	IL-3	PDGF-bb
HGF	IL-4	RANTES
ICAM-1	IL-6	SCF
IFN-α2	IL-7	SDF-1α
IFN-γ	IL-8	TNF-β
IGF-I	IP-10	TRAIL
IL-12(p40)	MCP-1	VCAM-1
IL-13	MCP-3	VEGF
IL-16	M-CSF	

Abbreviations: CTACK, cutaneous T cell-attracting chemokine; G-CSF, granulocyte colony stimulating factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; ICAM, intercellular adhesion molecule; IFN, interferon; MIP, macrophage inflammatory protein; MIF, macrophage migration inhibitory factor; MCP, monocyte chemotactic protein; M-CSF, monocyte colony stimulating factor; MIG, monokine induced by IFN-γ; NGF, nerve growth factor; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T-cell expressed and secreted; SCF, stem cell factor; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; VCAM, vascular cell adhesion molecule.

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Table 2. Clinicopathologic characteristics in patients with progression

	PD		*P*
	No (*n* = 26)	Yes (*n* = 6)	
Age, median (range)	52 (21–76)	59 (52–71)	0.049*
Sex			
Male	18 (90%)	2 (10%)	0.17
Female	8 (66.7%)	4 (33.3%)	—
Race			
White	24 (82.8%)	5 (17.2%)	0.48
Black	0 (0%)	1 (100%)	—
Hispanic	1 (100%)	0 (0%)	—
Asian	1 (100%)	0 (0%)	—
Smoking status			
Never smoker	12 (92.3%)	1 (7.7%)	0.17
Former smoker	8 (88.9%)	1 (11.1%)	—
Current smoker	6 (60%)	4 (40%)	—
Differentiation			
Well	1 (100%)	0 (0%)	0.70
Moderate	7 (87.5%)	1 (12.5%)	—
Poorly	11 (84.6%)	2 (15.4%)	—
Moderately well	1 (50%)	1 (50%)	—
Poorly moderately	2 (100%)	0 (0%)	—
T stage			
T_1–T_2	18 (86%)	3 (14%)	0.003
T_3–T_4	8 (73%)	3 (27%)	—
N stage			
N_{2B}	19 (90.5%)	2 (9.5%)	0.090
N_{2C}	5 (62.5%)	3 (37.5%)	—
N_3	1 (50%)	1 (50%)	—
EGFR†			
0	0 (0%)	0 (0%)	0.78
1+	4 (100%)	0 (0%)	
2+	5 (100%)	0 (0%)	
3+	37 (82.2%)	8 (17.8%)	
HPV‡			
HPV positive	12 (100%)	0 (0%)	0.008
HPV negative	8 (57%)	6 (43%)	

*P value from Wilcoxon rank sum test; all other P values were from Fisher's exact test.
†Available for 24 patients.
‡Available for 26 patients.

microspheres identified by a unique ratio of two fluorescent dyes to measure up to 100 biomolecules simultaneously. Performance characteristics are comparable with ELISA (21, 22), but these assays are less expensive and require less blood because of the multiplexed analysis (up to 50 biomarkers can be measured from 200 μL serum).

CAF concentrations were calculated based on a standard curve derived by performing six serial dilutions of a protein standard in assay diluent. Serum samples were tested in duplicate, and the mean value was used for analysis. If the mean of the duplicate values for all factors in an individual sample varied by >25%, the sample was retested. Individual factors were excluded from analysis if ≥50% of the samples were out of range or extrapolated. When out-of-range values were included in the analysis, the highest value (measured or extrapolated) across all samples was substituted for values above the upper limit of detection. For values below the lower limit of detection, half the lowest value was substituted. ELISA was used to measure insulin-like growth factor-I (Diagnostic Systems Laboratories, Inc.) and osteopontin (R&D Systems) according to the manufacturers' instructions. Both analytes were measured in duplicate, and the mean value was used for analysis.

Human papilloma virus

Tumor specimens from 26 of 47 patients were available for HPV DNA detection by *in situ* hybridization. Formalin-fixed paraffin-embedded tissue sections, 4 μm thick, were evaluated by *in situ* hybridization for HPV nucleic acids using the automated BenchMark per the manufacturer-recommended protocol (Ventana). Ventana inform HPV III Family 16 Probe (Ventana) was used, which contains a cocktail of labeled HPV genomic probes targeting the most common HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. Tumor cells were evaluated for positive nuclear expression with concurrent positive and negative controls reviewed. Of 26 tumors evaluated, 12 tested positive for oncogenic HPV DNA. There was no association between HPV status and clinical response to induction chemotherapy. However, patients with HPV-positive tumors had significantly longer progression-free survival (P = 0.012) and overall survival (P = 0.046; ref. 20).

Immunohistochemistry for EGFR

Immunohistochemistry was done on pretreatment tumor biopsies. Formalin-fixed paraffin-embedded tissue histology sections (5 μm thick) were deparaffinized, hydrated, and heated in a steamer for 30 minutes with Dako Target Retrieval (pH 6.0), for antigen retrieval (Dako North America, Inc.). Peroxide blocking was done with 3% H_2O_2 in methanol at room temperature for 15 minutes, followed by 10% fetal bovine serum in TBS-T for 30 minutes.

Slides were incubated with primary antibody against EGFR (total) clone:31G7 Zymed (Invitrogen, a division of Life Technologies Corporation) 1:100 at room temperature for 90 minutes and then probed for 30 minutes with the secondary antibody Envision Dual Link Plus (Dako). Staining was developed with 0.05% DAB+ (Dako) and counterstained with hematoxylin. Tumor staining for EGFR was quantified using a four-value intensity score (0, 1+, 2+, or 3+).

Statistical analysis

Progression-free survival and overall survival were calculated from the first day of induction therapy.

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Table 3. Baseline CAFs associated with PD

Covariate	Progression	*n*	Mean ± SD*	Median (min, max)*	*P*†
Eotaxin	Yes	6	4.8 ± 0.6	5.1 (4.1, 5.4)	0.016
	No	26	2.6 ± 2.0	2.6 (0, 5.9)	
Osteopontin	Yes	6	4.0 ± 2.1	4.5 (0, 6.2)	0.021
	No	26	2.0 ± 1.8	2.3 (0, 5.3)	
G-CSF	Yes	6	6.4 ± 1.5	5.9 (5.3, 9.2)	0.039
	No	26	4.2 ± 2.1	3.6 (2.3, 9.8)	
IL-4	Yes	6	2.7 ± 0.6	2.8 (1.9, 3.6)	0.040
	No	26	1.8 ± 0.9	1.7 (0.4, 3.4)	
SDF-1α	Yes	6	8.6 ± 0.2	8.6 (8.4, 8.9)	0.044
	No	26	8.0 ± 0.9	8.0 (6.5, 9.9)	
VEGF	Yes	6	6.0 ± 2.1	6.3 (3.7, 9.1)	0.049
	No	26	3.6 ± 2.5	3.2 (0.1, 8.3)	
GRO-α	Yes	6	7.1 ± 0.7	6.9 (6.2, 8.2)	0.049
	No	26	6.3 ± 0.7	6.3 (4.5, 7.9)	—
IL-8	Yes	6	5.5 ± 2.5	4.6 (3.2, 10)	0.050
	No	26	2.9 ± 2.6	2.4 (0.5, 9.1)	

*Values are pg/mL log 2 transformed.
†*P* value from the Wilcoxon rank sum test.

Fisher's exact test was used to assess the relationship between clinicopathologic features and PD. For baseline CAF analyses, CAF levels were log transformed (base 2) before analysis, and Wilcoxon rank sum test was used to compare CAF expression between patients with and without PD. For these exploratory analyses, a *P* value of ≤0.05 was considered significant. The eight CAFs differentially expressed between these two groups were then combined into a high-risk signature. The median of each CAF was used as the cutoff value for dichotomizing each marker. Using log-rank analysis, we compared progression-free survival in signature-positive patients (6–8 high-risk markers above the median) versus signature-negative patients (≤5 high-risk markers above the median). For modulation analysis, fold change from baseline to cycle 6 was compared by Wilcoxon rank sum test for each CAF in patients with and without PD. Unsupervised hierarchical clustering was done on log-transformed, mean-centered baseline CAF levels using Cluster and TreeView software (Eisen Lab) as previously described to identify subsets of patients with similar baseline CAF profiles.

All statistical analyses were done using SAS v9.2.0 (SAS Institute, Inc.) and graphed using the GraphPad Prism software v5.02 (GraphPad Software, Inc.).

Results

Clinical and pathologic comparison of patients with progression

Of the 47 clinical trial patients, 32 (including all six with PD) had baseline serum for CAF profiling and were included in biomarker analysis. There were no significant differences in demographics (sex, gender, race, smoking status) or baseline clinical characteristics (tumor differentiation, T stage, N stage, primary tumor location) between patients with and without biomarker data ($P \geq 0.17$ for all clinicopathologic variables by Fisher's exact *t* test).

Among the 32 patients available for CAF analysis, 20 (63%) were male and 19 (59%) were current or former smokers. All had locally advanced HNSCC, but there was a higher frequency of T_{1-2} disease than T_{3-4} (21 patients versus 11). A majority of patients had oropharyngeal primary tumors ($n = 27$). N_{2B} was the most common nodal stage ($n = 21$).

All patients had complete response, partial response, or stable disease following induction. Since completing definitive local therapy, 6 of the 32 patients have had disease progression. PD was associated with older age ($P = 0.049$), higher T stage ($P = 0.003$), and HPV-negative tumors ($P = 0.008$; Table 2). PD was not associated with response to induction, as two of six patients with PD had had complete response, three of six partial responses, and one of six stable disease immediately following chemotherapy. Qualitative EGFR levels by immunohistochemistry were not significantly correlated with stage, clinicoradiographic response, or disease progression.

Baseline serum CAFs associated with subsequent HNSCC progression

Baseline CAF levels ($\log_2$) were compared between patients with ($n = 6$) and without ($n = 26$) PD by Wilcoxon

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rank sum test. Eight CAFs were significantly higher in patients who later progressed ($P \leq 0.05$). These were VEGF, Gro-α, IL-4, IL-8, osteopontin, eotaxin, granulocyte colony-stimulating factor, and stromal cell–derived factor-1α (SDF-1α; Table 3; Fig. 1). Eotaxin and osteopontin levels were most strongly associated with PD (P = 0.016 and 0.021, respectively). Among these eight markers, there was a trend toward higher osteopontin levels in current smokers (median 3.8 pg/mL, range 0–6.2) versus never smokers or former smokers (median 2.3 pg/mL, range 0–5.3), although this was of borderline significance (P = 0.073 by Wilcoxon rank sum test).

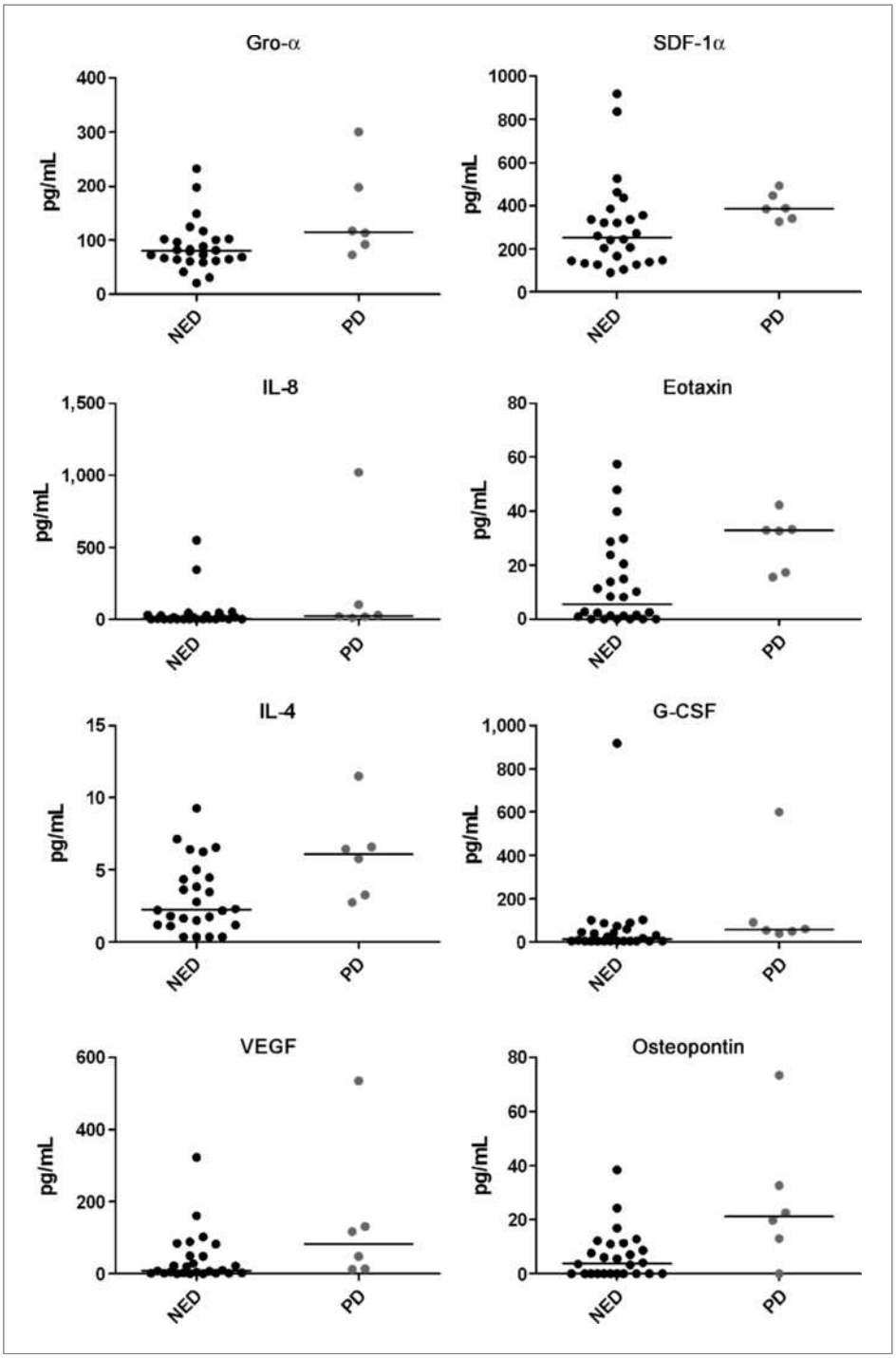

Figure 1. Comparison of baseline CAF levels in patients with and without PD for the eight high-risk markers. NED, no evidence of disease.

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High-risk baseline CAF signature predicts PD

We examined whether the combination of the eight CAFs that were differentially expressed at baseline into a high-risk signature would be more sensitive or specific for disease progression. For each patient, the numbers of CAFs in the high-risk signature that were above the median (across all baseline samples) was counted. PD was not seen in any patient with ≤5 high-risk CAFs above the median. However, among patients with 6 to 8 elevated high-risk CAFs, 6 of 13 (47%) of patients had PD (Fig. 2A). Elevation in 6 to 8 high-risk CAFs was 73% specific and 100% sensitive for PD. As expected, time to progression was also longer in patients with ≤5 elevated high-risk CAFs, as assessed by the log-rank test ($P = 0.001$; Fig. 2B).

Longitudinal increase in CAFs associated with PD

Previously, Allen et al. found that longitudinal changes in five NF-κB–mediated cytokines and growth factors (IL-6, IL-8, VEGF, HGF, and Gro-α) predicted worse outcome in patients with advanced or metastatic HNSCC (16). Here, we measured fold change of these and other factors from baseline to completion of induction therapy (6 weeks). CAF levels were available at both time points for 26 patients, including 4 who ultimately developed PD. As with the Allen et al. study, patients with PD had a significantly greater increase from baseline to cycle 6 in Gro-α (P value = 0.015). There was also a significant increase in βNGF (P = 0.039) and borderline significant increase in MCP3 (P = 0.053). There was no significant modulation of the other CAFs.

High-risk CAFs increased in HPV-negative patients with PD

Because all six progression events were in patients with HPV-negative tumors, we investigated whether CAF levels were either surrogates for HPV status or if they might add prognostic information beyond HPV status. HPV status was available for 26 of 32 patients with CAF profiles, of whom 12 (46%) were HPV positive and 14 (54%) HPV negative. No individual CAF was associated with HPV status.

We then performed hierarchical clustering of baseline CAF levels across all 32 patients and evaluated HPV between clusters. Clustering separated the patients into two distinct groups (11 patients in cluster 1; 21 in cluster 2; Fig. 3). Cluster 2 was characterized by higher levels of hypoxia-associated markers and contained all six patients with PD. However, there was a similar number of HPV-positive and HPV-negative patients in each cluster (Fig. 3), further suggesting that CAF expression was not primarily HPV driven.

High-risk CAF signature associated with PD among HPV-negative patients

Next, we evaluated whether individual CAF levels were different between six HPV-negative patients with PD and eight without. Osteopontin was statistically higher in HPV-negative patients with PD ($P = 0.04$). Gro-α ($P = 0.06$), IL-8 ($P = 0.08$), and VEGF ($P = 0.08$) were also elevated in HPV-negative patients with PD, although these did not reach statistical significance, possibly due to the small sample size.

Among the 14 HPV-negative patients, 8 were signature-positive (six to eight high-risk CAFs elevated), 6 of whom had PD. This suggests that the combination of the CAF high-risk signature with HPV status identifies patients at risk for PD beyond that of HPV status alone. Of note, there was no statistically significant association between smoking status and levels of high-risk CAFs among HPV-negative patients.

Discussion

The availability of multiplexing technologies now permits the simultaneous assessment of large numbers of biologically relevant proteins, such as cytokines, angiogenic factors and receptors, and soluble markers of hypoxia and immune activity using small amounts of serum or plasma. In this investigation, we studied serum biomarkers from a cohort of patients with locally advanced HNSCC from a phase II trial of induction

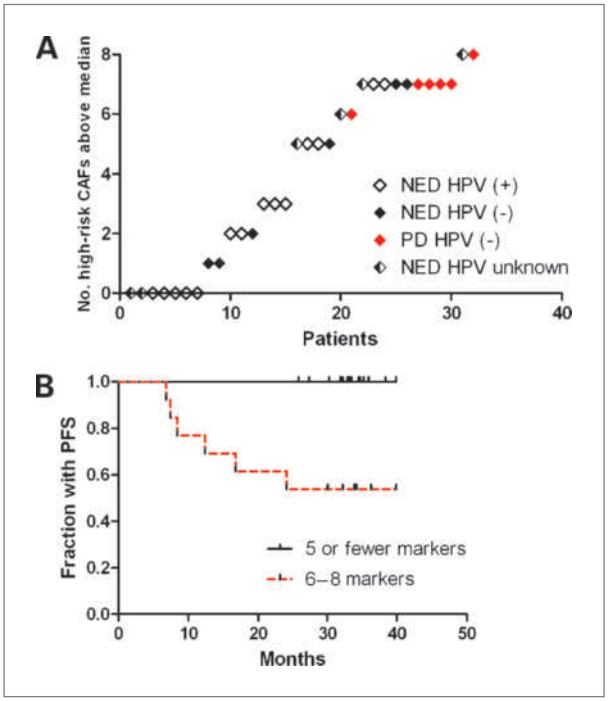

Figure 2. Elevations in ≥6 of 8 high-risk CAFs is associated with PD. The number of high-risk CAFs (VEGF, IL-4, IL-8, Gro-α, SDF-1α, G-CSF, osteopontin, and eotaxin) above the median was plotted for each patient (A). HPV status is indicated by open (HPV-positive) and closed symbols (HPV-negative). Patients with PD are indicated in red. Kaplan-Meier curve is shown for time to progression in patients with 0 to 5 versus 6 to 8 high-risk CAFs above the median (B). PFS, progression-free survival.

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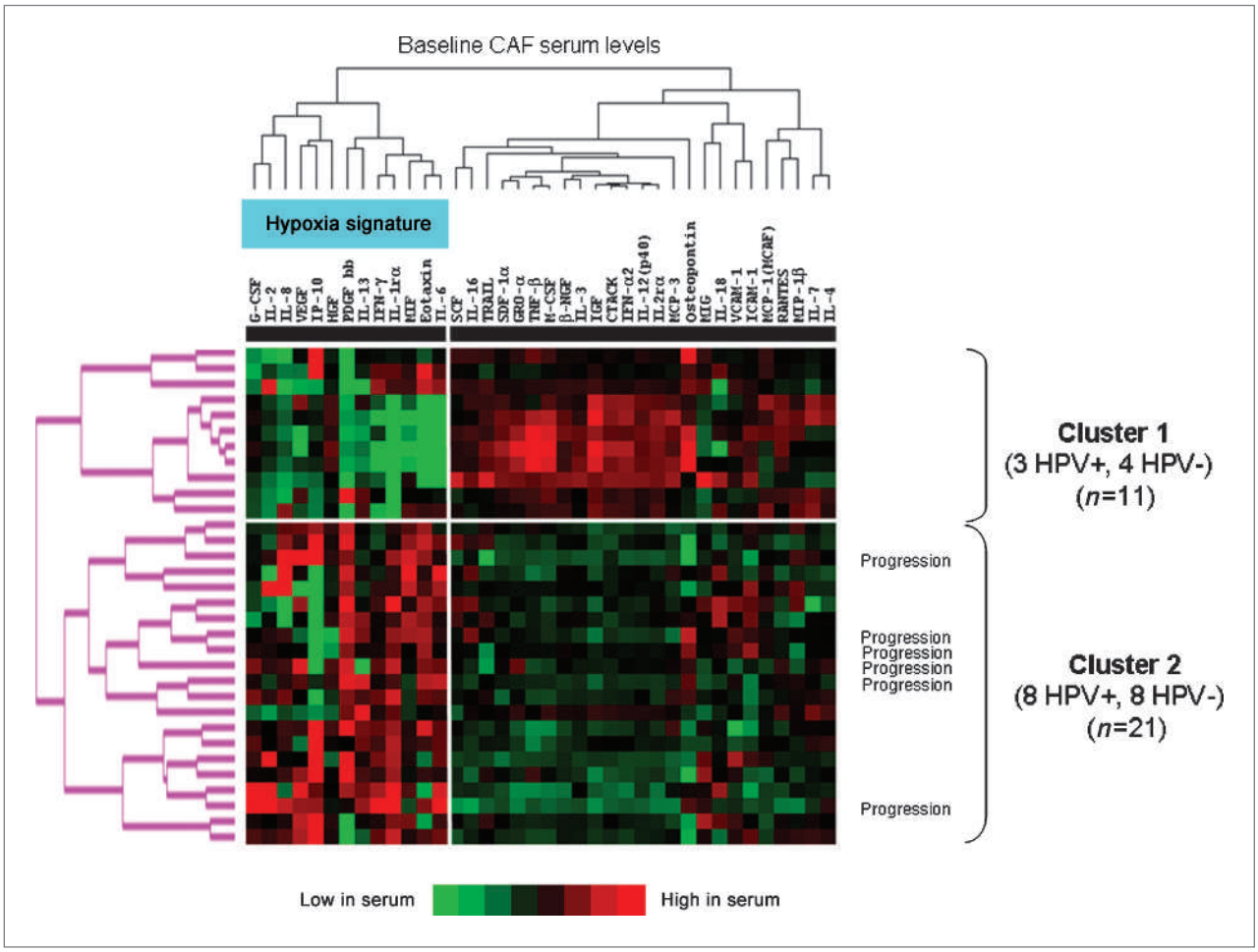

Figure 3. Unsupervised clustering identified two distinct subgroups that correlate with clinical outcome but not with HPV status. Relative serum levels of 38 baseline CAFs are shown (red, high protein levels; green, low protein levels). Each row contains CAF levels for an individual patient. Two distinct patient CAF profiles are identified (clusters 1 and 2), and all six patients with PD are in cluster 2, which is characterized by higher levels of hypoxia-associated factors, as indicated in the figure. There is no association between HPV status and clusters. See Table 1 legend for definitions of abbreviations.

therapy followed by definitive locoregional treatment. To our knowledge, this is the largest panel of serum markers to be studied in locally advanced HNSCC by multiplex bead analysis, and the only study in the setting of induction chemotherapy.

Our CAF analysis identified eight individual serum markers (VEGF, IL-4, IL-8, Gro-α, SDF-1α, granulocyte colony-stimulating factor, osteopontin, and eotaxin) higher in patients with subsequent PD. One of these (Gro-α) continued to increase longitudinally from baseline to week 6 to a greater extent in patients with PD. The combination of these 8 CAFs into a high-risk signature correlated better with PD than any individual CAF. Specifically, no patient with ≤5 elevated high-risk CAFs had PD, compared with PD in 6 of 13 patients with 6 to 8 high-risk CAFs. The combination of HPV negativity with high-risk serum CAF status further discriminated patients at greatest risk for PD than either one alone. This may reflect a difference in the biology of HPV-negative tumors or may be the result of the higher incidence of recurrence among this population.

Although this was an exploratory analysis, the results suggest that blood-based biomarkers may have a role as prognostic markers, beyond what is provided by currently available clinical or pathologic features. In this study, only age, T stage, and HPV-negative status were associated with a worse clinical outcome. Consistent with other studies, EGFR did not predict response or outcome, although there was a trend for patients with 3+ staining for EGFR to have shorter disease-free survival and overall survival despite treatment with cetuximab (23).

Among the individual CAFs associated with a worse prognosis, several have previously been associated with angiogenesis, tumorigenesis, and metastasis in HNSCC and other cancers (24–28). Among these, VEGF and IL-8 are the transcription products of genes included in the Harris metagene hypoxia signature, and SDF-1α is the ligand of a receptor (CXCR4) in the signature (10). The elevation of these factors in parallel suggests that we are able to detect the downstream effects of hypoxia in patient serum.

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Induction chemotherapy has been shown to decrease metastatic disease in HNSCC patients with locally advanced disease and is being studied in prospective randomized trials (29–31). However, previous studies have shown a correlation between EGFR inhibitor resistance and the upregulation of hypoxia-induced signaling by HIF1α and increased tumor angiogenesis, both of which are downstream effects of tumor hypoxia (7, 8). Therefore, our observation that patients with high levels of hypoxia-associated factors were more likely to relapse following induction therapy that included cetuximab may reflect the role of tumor hypoxia in therapeutic resistance and may be particularly relevant for regimens containing EGFR inhibitors.

Unlike the study of Allen at al. (16), which showed an association between low VEGF and shorter survival, in our study, patients with elevated VEGF were more likely to have PD. Both studies observed an association between increasing Gro-α and PD, although we did not see an increase in other NF-κB factors as they had observed. This discrepancy may be due to the different time course over which we assessed change in CAFs (6 weeks in our study versus 3-month intervals in theirs), differences in therapy, or small sample sizes. In addition, because all of the progression events occurred months to years after the end of induction therapy, it may have been too early to observe an increase in CAFs associated with PD. Because many of the CAF markers in the study can be modulated by inflammation and immune response, it is also possible that CAF levels were additionally influenced by hypoxia-independent mechanisms.

We believe that these results support further investigation of CAF profiling to predict clinical outcome in HNSCC patients and as a possible tool for surveillance, particularly among HPV-negative patients. In addition, CAF profiling would be a more practical tool for assessing tumor hypoxia than direct intratumoral measurement (6) and more accurate than indirect techniques such as immunohistochemistry of CA9 and HIF1α (32, 33). This is particularly true because CAF profiling is less invasive than direct tumor biopsy and, therefore, could be assessed longitudinally in the clinical setting with less morbidity and expense. Although this is a small, exploratory analysis, it shows the potential strength of blood biomarker profiling as a clinical tool for risk-stratifying patients. In addition, some of the serum markers in our high-risk signature could be targeted directly (e.g., VEGF, IL-4, and IL-8) or indirectly (e.g., Gro-α through inhibition of its receptor, CXCR2) by new targeted agents and may suggest a rationale for testing these drugs in patients with advanced HNSCC. We plan to validate our findings in an independent group of patients enrolled on a randomized, phase II trial of this induction regimen. The goal will be to validate the high-risk signature for its prognostic ability as well as to explore its predictive value.

Disclosure of Potential Conflicts of Interest

M.S. Kies received support for the phase II clinical trial described in this article from Bristol Myers Squibb Oncology Investigator Initiated trials program and Imclone Systems grant CS 2004-00011435 WC. No other conflicts of interest were disclosed.

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References

1. Jemal A, Siegel R, Ward E, Thun MJ, American Cancer Society. Cancer facts & figures. Atlanta: American Cancer Society; 2008.
2. Vikram B, Strong EW, Shah JP, Spiro R. Second malignant neoplasms in patients successfully treated with multimodality treatment for advanced head and neck cancer. Head Neck Surg 1984;6:734–7.
3. Adam MF, Gabalski EC, Bloch DA, et al. Tissue oxygen distribution in head and neck cancer patients. Head Neck 1999;21:146–53.
4. Harris AL. Hypoxia—a key regulatory factor in tumour growth. Nat Rev 2002;2:38–47.
5. Kaanders JH, Wijffels KI, Marres HA, et al. Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. Cancer Res 2002;62:7066–74.
6. Nordsmark M, Bentzen SM, Rudat V, et al. Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. Radiother Oncol 2005;77:18–24.
7. Viloria-Petit A, Crombet T, Jothy S, et al. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies *in vivo*: a role for altered tumor angiogenesis. Cancer Res 2001;61:5090–101.
8. Peng XH, Karna P, Cao Z, Jiang BH, Zhou M, Yang L. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1α signal pathways increases resistance to apoptosis by up-regulating survivin gene expression. J Biol Chem 2006;281:25903–14.
9. Denko NC, Fontana LA, Hudson KM, et al. Investigating hypoxic tumor physiology through gene expression patterns. Oncogene 2003;22:5907–14.
10. Winter SC, Buffa FM, Silva P, et al. Relation of a hypoxia metagene derived from head and neck cancer to prognosis of multiple cancers. Cancer Res 2007;67:3441–9.
11. Gourin CG, Xia ZS, Han Y, et al. Serum protein profile analysis in patients with head and neck squamous cell carcinoma. Otolaryngol Head Neck Surg 2006;132:390–7.
12. Linkov F, Lisovich A, Yurkovetsky Z, et al. Early detection of head and neck cancer: development of a novel screening tool using multiplexed immunobead-based biomarker profiling. Cancer Epidemiol Biomarkers Prev 2007;16:102–7.
13. St John MA, Li Y, Zhou X, et al. Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. Otolaryngol Head Neck Surg 2004;130:929–35.

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14. Wadsworth JT, Somers KD, Stack BC, Jr., et al. Identification of patients with head and neck cancer using serum protein profiles. Otolaryngol Head Neck Surg 2004;130:98–104.
15. Gourin CG, Moretz WH III, Weinberger PM, et al. Serum protein profile analysis following definitive treatment in patients with head and neck squamous cell carcinoma. Otolaryngol Head Neck Surg 2007; 133:1125–30.
16. Allen C, Duffy S, Teknos T, et al. Nuclear factor-κB-related serum factors as longitudinal biomarkers of response and survival in advanced oropharyngeal carcinoma. Clin Cancer Res 2007;13:3182–90.
17. Vidal L, Gillison ML. Human papillomavirus in HNSCC: recognition of a distinct disease type. Hematol Oncol Clin North Am 2008;22: 1125–42, vii.
18. Braakhuis BJ, Snijders PJ, Keune WJ, et al. Genetic patterns in head and neck cancers that contain or lack transcriptionally active human papillomavirus. J Natl Cancer Inst 2004;96:998–1006.
19. Slebos RJ, Yi Y, Ely K, et al. Gene expression differences associated with human papillomavirus status in head and neck squamous cell carcinoma. Clin Cancer Res 2006;12:701–9.
20. Kies MS, Holsinger FC, Lee JJ, et al. Induction chemotherapy and cetuximab for locally advanced squamous cell carcinoma of the head and neck: results from a phase II prospective trial. J Clin Oncol 2010;28:8–14.
21. Dupont NC, Wang K, Wadhwa PD, Culhane JF, Nelson EL. Validation and comparison of Luminex multiplex cytokine analysis kits with ELISA: determinations of a panel of nine cytokines in clinical sample culture supernatants. J Reprod Immunol 2005;66:175–91.
22. Giavedoni LD. Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using Luminex technology. J Immunol Methods 2005;301:89–101.
23. Morgillo F, Bareschino MA, Bianco R, Tortora G, Ciardiello F. Primary and acquired resistance to anti-EGFR targeted drugs in cancer therapy. Differentiation 2007;75:788–99.
24. Shang ZJ, Li JR, Li ZB. Circulating levels of vascular endothelial growth factor in patients with oral squamous cell carcinoma. Int J Oral Maxillofac Surg 2002;31:495–8.
25. Riedel F, Zaiss I, Herzog D, Gotte K, Naim R, Hormann K. Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma. Anticancer Res 2005;25:2761–5.
26. Hong SH, Ondrey FG, Avis IM, et al. Cyclooxygenase regulates human oropharyngeal carcinomas via the proinflammatory cytokine IL-6: a general role for inflammation? FASEB J 2000;14:1499–507.
27. Teknos TN, Cox C, Yoo S, et al. Elevated serum vascular endothelial growth factor and decreased survival in advanced laryngeal carcinoma. Head Neck 2002;24:1004–11.
28. Loukinova E, Dong G, Enamorado-Ayalya I, et al. Growth regulated oncogene-α expression by murine squamous cell carcinoma promotes tumor growth, metastasis, leukocyte infiltration and angiogenesis by a host CXC receptor-2 dependent mechanism. Oncogene 2000;19:3477–86.
29. Pignon JP, Bourhis J, Domenge C, Designe L. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. Lancet 2000;355:949–55.
30. Monnerat C, Faivre S, Temam S, Bourhis J, Raymond E. End points for new agents in induction chemotherapy for locally advanced head and neck cancers. Ann Oncol 2002;13:995–1006.
31. Cohen EE, Lingen MW, Vokes EE. The expanding role of systemic therapy in head and neck cancer. J Clin Oncol 2004;22:1743–52.
32. Koukourakis MI, Giatromanolaki A, Sivridis E, et al. Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. Clin Cancer Res 2001;7:3399–403.
33. Koukourakis MI, Giatromanolaki A, Sivridis E, et al. Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. Int J Radiat Oncol Biol Phys 2002;53:1192–202.

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