Coexpression of Hypoxia-inducible Factors 1α and 2α, Carbonic Anhydrase IX, and Vascular Endothelial Growth Factor in Nasopharyngeal Carcinoma and Relationship to Survival[1]

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ABSTRACT

Purpose: **Tumor hypoxia is known to be associated with resistance to chemotherapy, radiotherapy, and poorer survival. Recently, it is shown that hypoxia induces the expression of hypoxia-inducible factor-1α and 2α (HIF-1α and HIF-2α), which then up-regulates the expression of downstream genes such as carbonic anhydrase IX (CA IX) and vascular endothelial growth factor (VEGF).**

Experimental Design: **We examined the expression of HIF-1α, HIF-2α, CA IX, and VEGF by immunohistochemistry in nasopharyngeal carcinoma (NPC) biopsies from 90 consecutive patients recruited between 1994 and 1997 in a randomized controlled trial of chemoradiation in locally advanced NPC and investigated their relationship with survival.**

Results: **HIF-1α was expressed in 52 of 90 (58%), HIF-2α in 6 of 89 (7%), CA IX in 51 of 90 (57%), and VEGF in 54 of 90 (60%) of tumors. Tumor HIF-1α expression correlated significantly with that of CA IX ($P = 0.008$) and VEGF ($P = 0.003$). High tumor HIF-1α expression was associated with a trend for poor overall survival ($P = 0.06$). Tumors with a positive hypoxic profile (defined as high expression of both HIF-1α and CA9) were associated with worse progression-free survival ($P = 0.04$). Tumors with both hypoxic and angiogenic profile (defined as high VEGF expression) were associated with a worse progression-free survival ($P = 0.0095$).**

Conclusion: **Overexpression of HIF-1α, CA IX, and VEGF is common in NPC, which is probably related to hypoxia up-regulated expression involving a HIF-dependent pathway, and is associated with poor prognosis. Targeting the hypoxia pathway may be useful in the treatment of NPC.**

INTRODUCTION

In the West, NPC[3] occurs sporadically and, histologically, usually belongs to the WHO type I squamous cell carcinoma, which is associated with alcohol and smoking. In many parts of Asia, including Southern China and Southeast Asia, NPC is an endemic disease with incidence rates of 15–50 per 100,000 (1). Histologically, these usually belong to WHO types II and III, nonkeratinizing and undifferentiated carcinoma. Unlike WHO type I, types II and III have no association with alcohol and smoking but are strongly associated with the EBV.

The primary treatment for nonmetastatic NPC is radical external RT. More accurate tumor localization by computed tomography and better RT technique have contributed to improvement in local control of this disease, with a local failure-free rate of ~80% (2). However, patients with locoregionally advanced disease have significant rates of both local recurrences and particularly distant metastases after RT alone. Hence, new therapeutic strategies are urgently needed. Recent evidence from two prospective randomized trials supports the routine use of concurrent cisplatin-radiotherapy in these patients (3, 4).

Tumor hypoxia has long been known to be associated with resistance to chemotherapy and radiotherapy as well as a more malignant tumor phenotype with increased invasiveness, metastases, and poorer survival (5–7). The development of simple and reliable tests to estimate tumor hypoxia would be of clinical importance for the identification of subgroups of patients that could benefit from hypoxia targeting therapeutic strategies. Previous clinical studies of tumor hypoxia concentrated on direct measurement with a polarographic electrode or by injection of a hypoxia labeling marker, such as pimonidazole, into the patient's blood before biopsy and subsequent detection of the marker by immunohistochemistry. The recent development of scintigraphic and magnetic resonance imaging of hypoxia-labeling markers are promising new approaches. In head and neck

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[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; HIF, hypoxia-inducible factor; CA IX, carbonic anhydrase IX; VEGF, vascular endothelial growth factor; RT, radiotherapy; OS, overall survival; PFS, progression-free survival.

squamous cell cancer, using direct measurement of tumor pO_2, a low pretreatment pO_2 has been shown to predict poor response to radiation and shorter survival (8, 9). In NPC, using the hypoxia imaging agent fluorine-18 fluoromisonidazole with positron emission tomography system, tumor hypoxia was demonstrated in 100% of primary tumor and 58% of cervical lymph nodes metastases (10). However, these techniques can only be applied *in vivo* and on a prospective basis. In search of a simple test that would detect evidence of hypoxia even on archival tissue material, immunohistochemical detection of proteins induced by clinical relevant levels of hypoxia represents an appealing option. Such intrinsic marker of hypoxia would have the advantage of being assessable on routine clinical biopsies without the need for specialist equipment or administration of exogenous hypoxia markers.

The transcriptional complex HIF-1 plays a pivotal role in essential adaptive responses to hypoxia, and its expression increases exponentially with decreases in levels of cellular oxygen. HIF-1 has emerged recently as an important mediator of gene expression patterns in many tumors (11). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. HIF-1β is constitutively expressed, whereas HIF-1α is protected from ubiquitination and proteasomal degradation under hypoxic conditions (12). Recently, another member of the family showing close sequence homology and similar properties to HIF-1α has been described and is named HIF-2α (also known as endothelial PAS domain protein-1; Ref. 13).

CA IX is a novel member of the CA family that codes for a transmembrane glycoprotein with a suggested function in maintaining the acid-base balance and intercellular communication (14). CA IX can confer a variety of features of the transformed phenotype when transfected into NIH 3T3 cells (15). VEGF is one of the most well-studied markers of tumor angiogenesis, and its expression has been shown to be of prognostic significance in most human tumors studied.

Recently, it has been shown that hypoxia induces the expression of HIF-1α, which then up-regulates the expression of downstream genes *CA IX* and *VEGF* (16–18). In cervical cancer, CA IX expression was demonstrated to correlate with tumor hypoxia as measured by needle electrode (19). HIF-1α expression was shown to overlap with the hypoxia-labeling marker EF5 in cervical cancer xenograft (20). HIF-1α and CA IX are thus potential endogenous markers of tissue hypoxia.

This study was undertaken with the aim to establish the expression pattern of HIF-1α, HIF-2α, CA IX, and VEGF in NPC biopsies and to correlate the level of expression with clinicopathological characteristics and survival outcome.

MATERIALS AND METHODS

Patients and Tissues. Formalin-fixed, paraffin-embedded biopsy tissues of 90 consecutive NPC patients recruited between 1994 and 1997 in a randomized controlled trial of chemoradiation in locally advanced NPC were retrieved. Patients with biopsy-proven, previously untreated NPC with Ho's N2 or N3 stage, or N1 stage with lymph node size ≥4 cm (Ho's staging; Ref. 1), and without distant metastases (M0), were eligible for the trial. Eligible patients were randomized to receive either standard radiotherapy alone or the same radiotherapy given concurrently with weekly 40 mg/m^2 cisplatin for up to 8 weeks (4). After completion of treatment, patients were followed up every 8 weeks during the first year, every 12 weeks for the second year and third years, and every 16 to 24 weeks thereafter. Patients who developed local or distant recurrence were subjected to any treatment considered appropriate in the opinion of the attending physician including surgery, chemotherapy, or radiotherapy. Informed consent for immunohistochemical study of biological markers was obtained at the time of biopsy. All biopsies were taken via a nasopharyngeal endoscope for diagnostic purposes before the start of treatment. Flanking sections from each tumor biopsy were studied by immunohistochemistry for the expression of HIF-1α, HIF-2α, CA IX, and VEGF.

Immunohistochemistry. The HIF-1α, HIF-2α, CA IX, and VEGF proteins in tissue sections were detected using the murine monoclonal antibodies ESEE 122 (21), EP 190b (21), M75 (22), and VG1 (23), respectively, according to methodology described previously (16, 21, 23, 24).

HIF-1α and HIF-2α. Staining was performed on 4–5 μm paraffin sections. The slides were first placed in a 60°C oven for 15 min. They were then deparaffinized in Citroclear twice for 10 min and rehydrated through a series of graded alcohols and distilled water. After being placed in Tris buffered saline (TBS) buffer for 5 min, the slides were transferred to a jar containing 1 mM EDTA (pH 8.0) buffer and placed in a preheated 60°C water bath for overnight incubation to achieve antigen retrieval. To block endogenous peroxidase activity, DAKO peroxidase block solution was applied for 5 min. After two washes in TBS buffer, 0.2% Triton X-100 in TBS was applied for 10 min. For HIF-1α, the primary antibody ESEE 122 was applied at 1:40 dilution for 30 min. For HIF-2α, the primary antibody EP 190b was applied without dilution for 30 min. Secondary labeled polymer from the Envision HRP Kit (DAKO) was applied for 30 min. The peroxidase reaction was developed using diaminobenzidine chromogen kit from DAKO for 10 min. After washing, the slides were lightly counterstained with Hematoxylin and mounted.

CA IX. Sections were deparaffinized and rehydrated. Endogenous peroxidase was quenched with DAKO peroxidase block solution applied for 5 min. Normal human serum at 10% in TBS was then applied for 15 min. After knocking off excess blocking serum from the slides, the primary antibody M75 was applied at 1:50 for 30 min. Secondary polymer from the Envision HRP kit (DAKO) was applied for 30 min. Diaminobenzidine (DAKO) was applied for 8 min. The slides were then counterstained with hematoxylin and mounted.

VEGF. The paraffin sections were baked in a 60°C oven for 15 min before deparaffinization. Antigen retrieval was achieved by pressure cooking in Tris-EDTA (pH 9.0) buffer (preparation: 12.2 g of Tris base, 1.48 g of EDTA in 2 liters of distilled water) for 3 min. DAKO peroxidase block solution was applied for 5 min. Primary antibody VG1 was applied in 1:10 dilution for 30 min. Secondary polymer from the Envision HRP kit (DAKO) was applied for 30 min, followed by diaminobenzidine (DAKO) for 5 min. The slides were counterstained with hematoxylin and mounted.

For all of the above staining, positive control slides from a tissue block with known positive staining for the respective

primary antibody was included with each run. Negative control was achieved by substituting the primary antibody with TBS. In the case of VEGF, the universal presence of serum (which contains VEGF) in the blood vessel lumens of each section also served as a good internal positive control.

Scoring Method. All slides were evaluated independently by two investigators (E. P. H. and F. P.) who were blinded to the patient's clinical data. The difference in scores between the two observers was resolved at a conference microscopy. Each slide was examined at low (×40) and high (×250) power to study both the staining pattern and distribution. The staining pattern of individual cells was classified into membranous, nuclear, or cytoplasmic. The percentage of tumor cells showing positive staining for each antibody under study was scored. Staining intensity was not incorporated in our scoring method because we noted that it was more or less constant. The staining pattern of stromal cells and normal epithelium in the same tissue section was also assessed. The following grading system was adopted to score the number of positive stained macrophages in the tumor or stroma with HIF-2α: 0, none seen in section; +/−, very occasional single cell positive: +, few positive cells either in foci or scattered; ++, moderate numbers either in foci or scattered; +++, large numbers of positive cells.

Statistical Analysis. All statistical analysis and graphs were performed with the statistical package SPSS Release 9.0.0 (SPSS, Inc., Chicago, IL). Correlation among the markers was analyzed using Spearman's correlation. Association between HIF-1α, HIF-2α, CA IX, and VEGF expression and the various clinicopathological parameters was analyzed using χ^2 test. Overall survival was defined from the day of randomization to the day of death or last follow-up. Progression-free survival was defined from the day of randomization to the day of progression or last follow-up. Univariate analysis of overall survival and progression-free survival was performed by the Kaplan-Meier method and log-rank test. The Cox proportional hazards model was used for multivariate analysis. For all tests, a two-sided $P < 0.05$ was considered significant.

Table 1 Patient demographics

Characteristics		No. of patients (n = 90)
Age (yr)		
Median	45	
Range	21–67	
<40		25
≥40		65
Sex		
Male		73
Female		17
Histology		
Poorly differentiated squamous carcinoma (WHO II)		3
Undifferentiated carcinoma (WHO III)		87
Ho's T stage		
1		20
2		48
3		22
Ho's N stage		
1		7
2		51
3		32
LN size		
>3 cm		29
≤3 cm		61
Ho's overall staging		
II		7
III		51
IV		32
Treatment arm		
Chemoradiotherapy		48
RT alone		42
Duration of follow-up (yr)		
Median	4.13	
Range	0.52–6.21	
Failure pattern		
Local recurrence	4	
Distant metastases	30	
Death	26	

RESULTS

Study Cohort. The clinicopathological characteristics of the 90 NPC patients are presented in Table 1. The median duration of follow-up for this cohort was 4.13 years (range, 0.52–6.21 years) at the time of analysis. To date, there have been 4 local recurrences, 30 distant metastases, and 26 deaths.

Expression Pattern. Table 2 summarizes the expression pattern of HIF-1α, HIF-2α, CA IX, and VEGF in the tumor, stroma, and normal epithelium in the NPC biopsy tissues.

HIF-1α. The tumor cells showed typical nuclear staining of HIF-1α in 52 of 90 (58%) of cases. Overall, the percentage of positive cells in tumor ranged from 0 to 90% (median, 1%; mean, 8.4%; Fig. 1*A*). In the stroma, 12 cases (13%) showed nuclear staining. The normal epithelium showed focal staining in 16 of 60 (27%) cases, including 7 cases of nuclear stain, 4 cases of cytoplasmic stain, and 5 cases of mixed nuclear and cytoplasmic stain pattern.

HIF-2α. The tumor cells were mostly negative and only showed focal nuclear stain in 6 of 89 (7%) cases. Scanty focal cytoplasmic stain of tumor was noted in 6 of 89 (7%) cases (of which 3 cases showed focal nuclear stain as well). Focal cytoplasmic staining was observed in the stroma of 12 of 89 (13%) cases. The normal epithelium showed focal staining in 9 of 57 (16%) cases (cytoplasmic, 6; nuclear, 2; and mixed cytoplasmic and nuclear, 1).

In contrast, a striking pattern of HIF-2α expression in tissue infiltrating macrophages was observed in both the tumor and stroma (summarized in Table 3). We generally observed a higher number of HIF-2α-expressing macrophages in the stroma than the corresponding tumor.

CA IX. In the tumor, 51 of 90 (57%) cases showed the characteristic membranous staining pattern of CA IX. Overall, the percentage of positively stained tumor cells ranged from 0 to 80% (median, 1%; mean, 7.5%; Fig. 1*B*). The tumor typically showed a periluminal and perinecrotic distribution of membranous staining for CA IX. A tumor nuclear staining pattern was observed in only 4 of 90 (5%) cases. Only 2 cases showed positive staining of CA IX in stroma, including 1 nuclear and 1 cytoplasmic pattern.

A distinctive pattern of focal membranous staining of CA IX in the basal cells was observed in 30 of 57 cases of normal epithelium (including both respiratory epithelium and squamous

Table 2 Expression pattern of HIF-1α, HIF-2α, CA IX, and VEGF in NPC biopsies

The number of positive staining over total number of evaluable cases is shown. A positive staining pattern was further classified into membranous, nuclear, or cytoplasmic.

	Tumor		Stroma		Normal epithelium	
HIF-1α	Nuclear	52/90 (58%)	Nuclear	12/90 (13%)	Nuclear	7
					Cytoplasmic	4
					Nuclear and cytoplasmic	5
					All = 16/60 (27%)	
HIF-2α	Nuclear	6/89 (7%)	Cytoplasmic	12/89 (13%)	Nuclear	2
	Cytoplasmic	6/89 (7%)			Cytoplasmic	6
					Nuclear and cytoplasmic	1
					All = 9/57 (16%)	
CA IX	Membranous	51/90 (57%)	Membranous	1/90 (1%)	Membranous	30
	Nuclear	4/90 (4%)	Nuclear	2/90 (2%)	Cytoplasmic	2
					All = 32/57 (56%)	
VEGF	Cytoplasmic	54/90 (60%)		Negative	Cytoplasmic	50/66 (76%)

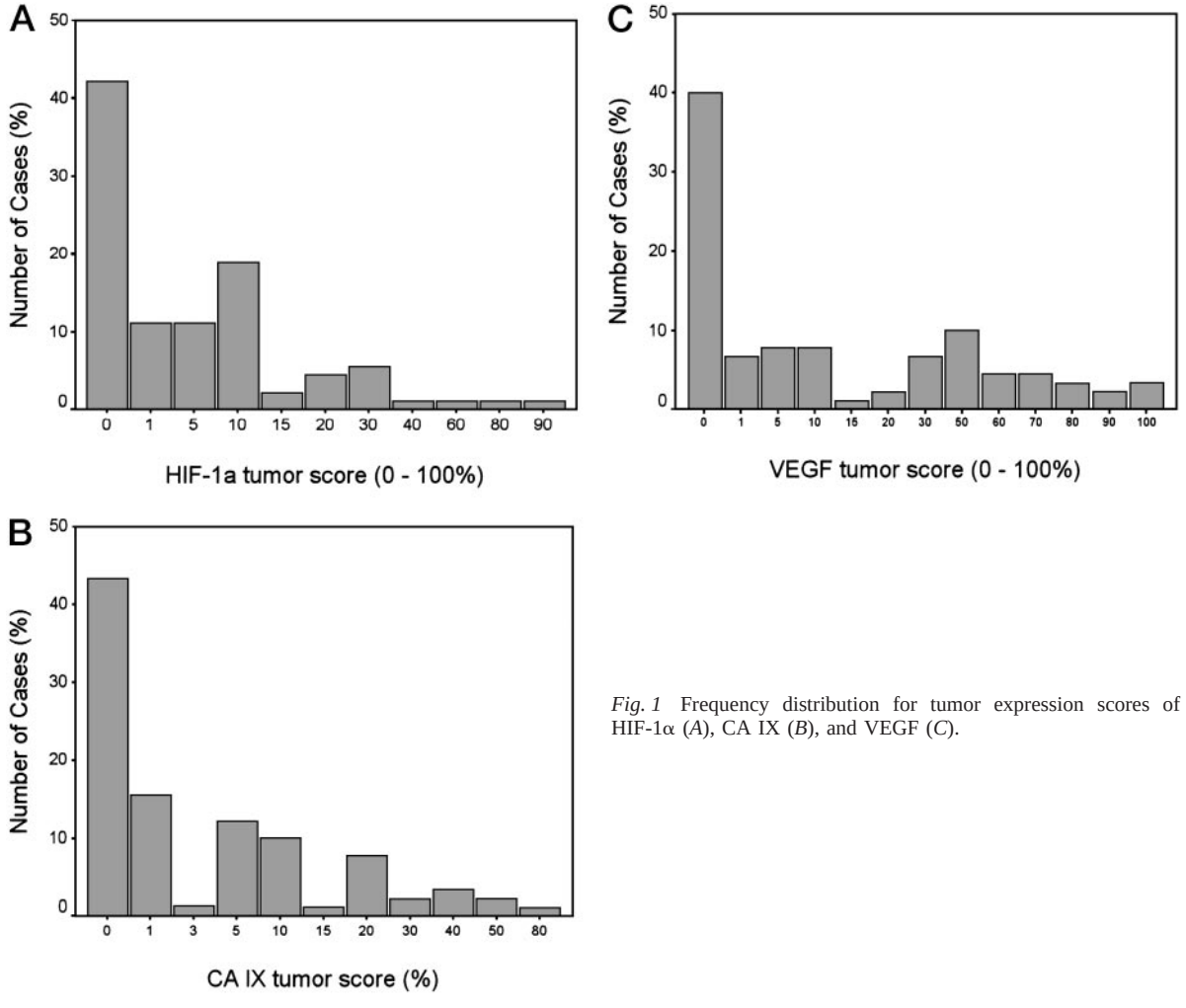

Fig. 1 Frequency distribution for tumor expression scores of HIF-1α (*A*), CA IX (*B*), and VEGF (*C*).

epithelium) and another 2 cases of normal epithelium revealed a focal nuclear staining. Additionally, paratumoral dysplastic lesions frequently demonstrated focal positive staining, independently of tumor stain.

VEGF. The near universal presence of serum within the blood vessel lumens and in the interstitial space at area of broken tissues served as strong internal positive control. For the same reason, only areas of intact tumor cells were scored. In the

Table 3 Expression pattern of HIF-2α in tissue-infiltrating macrophages[a]

Grading[b]	Negative	+/−	+	++	+++	Total (*n*)
Macrophage in tumor	65	14	9	0	1	89
Macrophage in stroma	31	26	20	9	3	89

[a] The number of cases in each grade is shown.

[b] Grading: +/−, very occasional single cell positive; +, few positive cells either in foci or scattered; ++, moderate numbers either in foci or scattered; +++, large numbers of positive cells.

tumor, 54 of 90 (60%) cases showed cytoplasmic staining for VEGF. Overall, the percentage of positive cells in tumor ranged from 0 to 100% (median, 5%; mean, 23%; Fig. 1*C*). In the normal epithelium, 50 of 66 (76%) cases showed cytoplasmic staining of VEGF. Of special note, in the respiratory epithelium it was the upper ciliated cells that showed positive cytoplasmic stain. In contrast, the oral squamous type epithelium was less often stained, and it was the basal cells that showed positive cytoplasmic stain. In general, the staining intensity of the tumor was weaker than or equal to that in the corresponding normal epithelium.

Coexpression of Markers. Because serial flanking sections from each tumor were studied for all four markers, we had the opportunity to observe whether there was any pattern of coexpression among the markers. A frequent observation was that if the same tumor stained positive for HIF-1α, it was also positive for CA IX and VEGF as well, although at the microscopic level the positively stained cells of each marker usually did not overlap. This is well illustrated in the photomicrographs in Fig. 2, *a–c*, which demonstrated the expression of the three markers, respectively, in the same flanking tumor section. Indeed, by Spearman's rank sum test, tumor HIF-1α expression correlated significantly with that of CA IX expression (correlation coefficient, 0.28; $P = 0.008$) and also with VEGF expression (correlation coefficient, 0.31; $P = 0.003$), although the correlation coefficients were small. However, tumor CA IX expression did not correlate with VEGF expression (correlation coefficient, −0.014; $P = 0.899$; Table 4).

Expression in Tumor and Normal Epithelium. There was a significant correlation between HIF-1α expression in tumor and in the corresponding normal epithelium in the same section of the tumor (correlation coefficient, 0.40; $P = 0.0023$). A strong correlation between VEGF expression in tumor and in the corresponding normal epithelium was also evident (correlation coefficient, 0.45; $P = 0.0003$). On the other hand, no significant correlation between CA IX expression in tumor and in the corresponding normal epithelium was found (correlation coefficient, 0.09; $P = 0.49$).

Clinical Correlation and Survival Analysis. For clinical correlation and survival analysis, a cutoff expression level of 5% was used throughout for HIF-1α, CA IX, and VEGF. Tumor with <5% positive cells for the marker was defined as low expression. Tumor with 5% or more positive cells was defined as high expression. This 5% level was chosen based on both the median score and a practical consideration so that borderline staining would be regarded as low expression. For HIF-2α, because the tumor cells only showed infrequent staining, we used the score of positively stained macrophages in tumor or stroma for analysis (negative, 0; positive, +/−, +, ++, or +++).

There was no significant association of the expression of HIF-1α, HIF-2α, CA IX, or VEGF with any of the following clinicopathological parameters: gender, histology type, Ho's T stages, Ho's N stages, Ho's overall stages, lymph node size, treatment arm, local recurrences, and distant metastases ($P > 0.05$ by χ^2 test, data not shown).

By univariate analysis, high tumor HIF-1α expression was associated with poor OS, but this only reached borderline significance (log-rank test, $P = 0.06$; Fig. 3*A*) and a nonsignificant trend of poor PFS (log-rank test, $P = 0.11$). On the other hand, no significant association between HIF-2α, CA IX, or VEGF expression with respect to OS or PFS was found (Table 5). None of the other parameters, including gender, age, histology type, Ho's T stages, Ho's N stages, and lymph node size, reached statistical significance by univariate analysis for OS or PFS (data not shown).

Overall Effect of Tumor Hypoxic and Angiogenic Profile on Survival. Because hypoxia induces the expression of HIF-1α, which then up-regulates the expression of downstream genes *CA IX* and *VEGF* (16–18), HIF-1α and CA IX has been suggested to be endogenous markers of tumor hypoxia (19, 20). We therefore proposed to define a model of tumor hypoxic and angiogenic profile based on the coexpression of HIF-1α, CA IX, and VEGF. We defined tumors with a high expression of both HIF-1α and CA IX as having a hypoxic profile and those with high VEGF expression as possessing an angiogenic profile. By the above definition, 20 of the 90 patients had a hypoxic tumor profile. This group was found to have a significantly worse PFS (log-rank test, $P = 0.04$; Fig. 3*B*) and a marginally significant worse OS ($P = 0.06$). Ten of the 90 patients had both a hypoxic and angiogenic tumor profile by the above definition. This group was found to have a significantly worse PFS (log-rank test, $P = 0.0095$; Fig. 3*C*) but not a worse OS ($P = 0.1$; summarized in Table 5).

Multivariate Analysis. The analysis of PFS in the original Phase III randomized trial of concurrent chemoradiation *versus* radiotherapy alone has been reported recently. Although the PFS was not significantly different between the two treatment arms in the overall comparison, it was significantly prolonged in the subgroup of patients with advanced tumor and node stages (4). Therefore, we incorporated the treatment arm as a potential prognostic and/or confounding factor in the multivariate analysis. In Cox's proportional hazard model, we included HIF-1α, tumor hypoxic profile, and tumor hypoxic and angiogenic profile together with the treatment arm as covariates. The HIF-1α or tumor hypoxic profile was not significant independently of the treatment arm. Only the hypoxic and angiogenic profile retained its significance independently of the treatment arm on PFS ($P = 0.022$ for hypoxic and angiogenic profile; $P = 0.027$ for the treatment arm).

DISCUSSION

This is, to our knowledge, the first study to determine the expression pattern of the recently described hypoxia markers HIF-1α, HIF-2α, and CA IX in NPC. It is also the first attempt

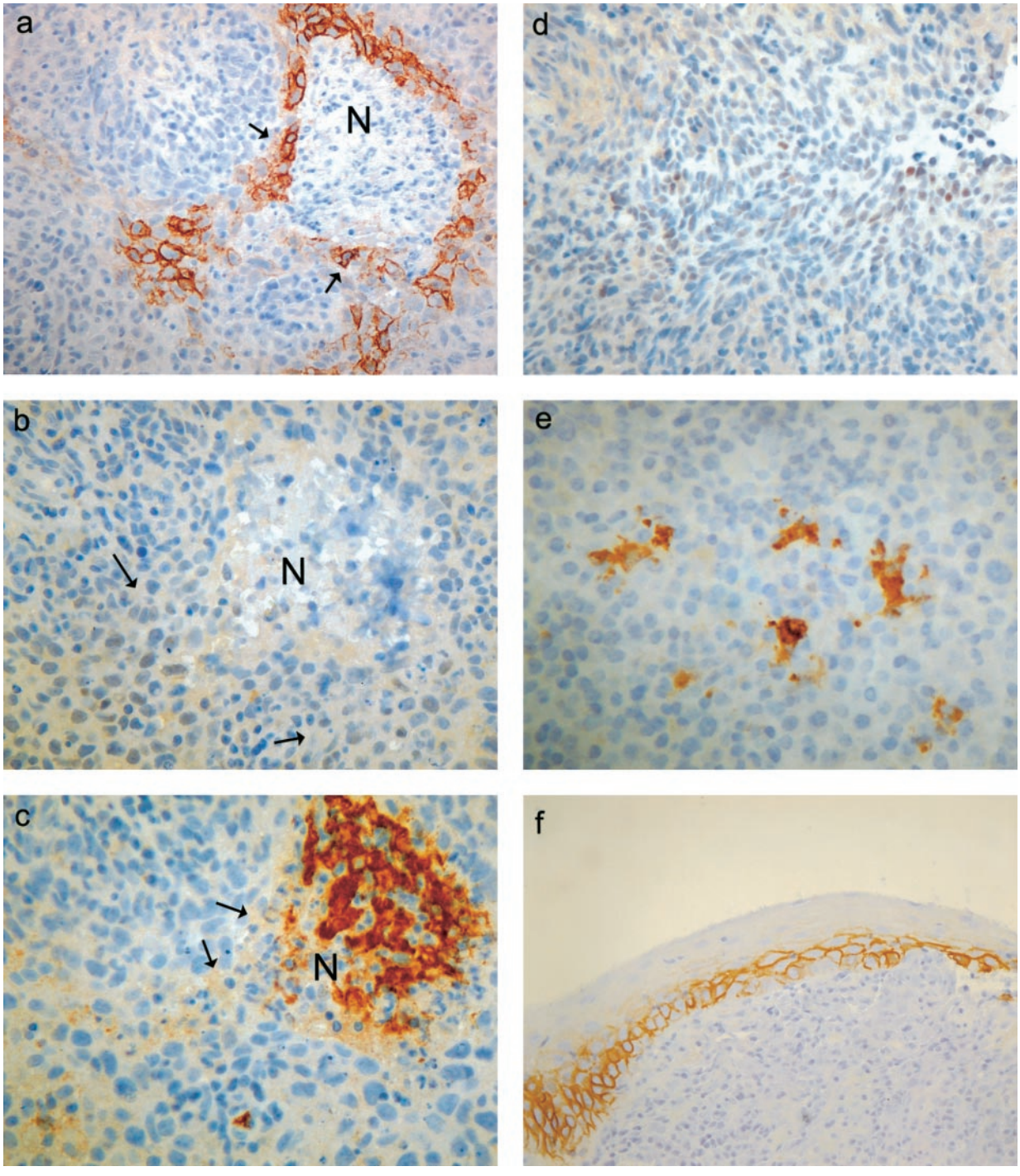

Fig. 2 Infiltrating NPCs. Expression pattern of CA IX (*a*), HIF-1α (*b*), and VEGF (*c*) in tumor cells in serial flanking sections. Note the perinecrotic distribution of positively stained tumor cells (*arrows*). *N,* necrosis). ×250. *d,* expression of HIF-1α in tumor cells showing nuclear staining pattern. ×200. *e,* expression of HIF-2α in tissue-infiltrating macrophages. ×400. *f,* expression of CA IX in the basal cells of normal epithelium in NPC biopsy. ×200. All immunoperoxidase staining was performed with diaminobenzidine, hematoxylin counterstain.

to study the coexpression of all three hypoxia markers HIF-1α, HIF-2α and CA IX together with the angiogenesis marker VEGF simultaneously in the same tissue sample from a cohort of NPC patients with long-term follow-up and survival data.

We have found that HIF-1α was expressed in 58%, HIF-2α in 7%, CA IX in 57%, and VEGF in 60% of tumors from NPC biopsy specimens. Our observations are similar to the findings by other investigators in other human tumors (21, 25, 26). Taken

Table 4 Correlations between HIF-1α, CA IX, and VEGF expression in tumors

	Spearman's rho	HIF-1α	CA IX	VEGF
HIF-1α	Correlation coefficient	1	0.280	0.313
	Significance (*P*)	0	0.008[a]	0.003[a]
CA IX	Correlation coefficient	0.280	1	−0.014
	Significance (*P*)	0.008[a]	0	0.899
VEGF	Correlation coefficient	0.313	−0.014	1
	Significance (*P*)	0.003[a]	0.899	0

[a] Correlation is significant at the 0.01 level (two-tailed).

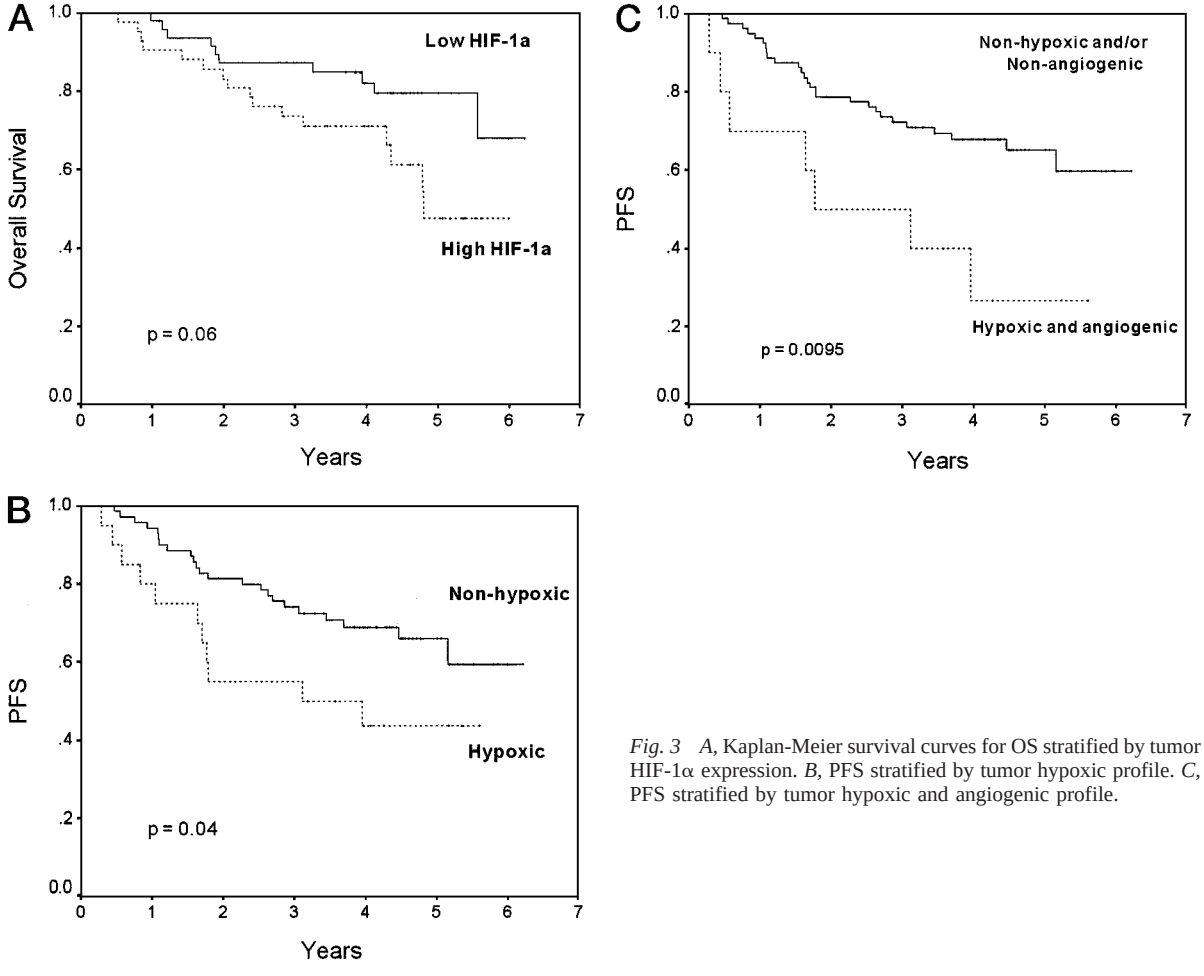

Fig. 3 *A,* Kaplan-Meier survival curves for OS stratified by tumor HIF-1α expression. *B,* PFS stratified by tumor hypoxic profile. *C,* PFS stratified by tumor hypoxic and angiogenic profile.

together, these results suggest that overexpression of the hypoxia markers HIF-1α, HIF-2α, CA IX, and the angiogenic factor VEGF are general phenomena in human malignancy. Furthermore, we have found that tumor expression of HIF-1α correlates significantly with that of CA IX and with VEGF. This coexpression of HIF-1α with CA IX, and of HIF-1α with VEGF, in clinical tumor is in concordance with previously described *in vitro* findings in cell lines. The *in vitro* hypoxia experiments have already shown that both *CA IX* and *VEGF* are downstream genes of HIF-1α, and that their protein levels are up-regulated by HIF-1α under conditions of hypoxia (16, 18).

On the other hand, we found that tumor CA IX expression did not correlate with VEGF expression. The reason may be that VEGF and CA IX transcripts have a short half-life after reperfusion and because VEGF protein is secreted and has a rapid clearance, VEGF will be subjected to rapid change with changes in intermittent oxygenation. In contrast, the CA IX protein has a half-life of over 24 h[4] and would reflect an integration of hypoxia over longer periods of time. Both are regulated by HIF but may reflect different time courses of events.

[4] K. Turner and A. Harris, submitted for publication.

Table 5 Univariate analysis of OS and PFS for hypoxia marker(s)

	OS			PFS		
	Risk ratio	95% CI[a]	*P*	Risk ratio	95% CI	*P*
HIF-1α	2.12	0.96–4.70	0.06	1.72	0.87–3.39	0.12
CA IX	1.39	0.64–3.01	0.40	1.28	0.65–2.52	0.48
VEGF	1.12	0.52–2.41	0.78	1.52	0.76–3.04	0.24
HIF-1α + CA IX (hypoxic profile)	2.13	0.95–4.80	0.06	2.06	1.00–4.23	0.04[b]
HIF-1α + CA IX + VEGF (hypoxic and angiogenic profile)	2.22	0.84–5.88	0.10	2.87	1.25–6.59	0.01[b]

[a] CI, confidence interval
[b] $P < 0.05$.

Another interesting finding was that HIF-1α was expressed in 27%, HIF-2α in 16%, CA IX in 56%, and VEGF in 76% of the (morphologically) normal epithelium present in the same section of the tumor. Moreover, the expression of HIF-1α and VEGF in tumor correlates significantly with its expression in the corresponding normal epithelium. It was often observed that the positively stained normal epithelium was close or adjacent to the underlying tumor. It should be pointed out that the morphologically normal epithelium adjacent to tumor was probably not equivalent to their counterpart in a healthy person without NPC. We have not studied, and it would be interesting to know, the expression pattern of these markers in the normal nasopharyngeal epithelium of a healthy individual. Previous studies on the expression of HIF-1α and HIF-2α in normal human tissue and cancer demonstrated that these markers were mostly negative in the normal tissues but were overexpressed in most human tumors (21, 25). The expression of CA IX in normal adult tissues was shown to be restricted to certain highly specialized cells, *e.g.,* bile duct and gastric mucosa (26). Our observations suggest that the normal epithelium near the tumor probably share the same microenvironmental hypoxia so that they overexpress the same marker. The growth of tumor probably created local circulatory shunts, and/or caused increased O_2 consumption diverting oxygen away from normal tissues.

We found a striking pattern of HIF-2α expression in tissue-infiltrating macrophages within both tumor and stroma of NPC. Talks *et al.* (21) also described a similar observation in several other tumor types. Morphological identification of these cells as macrophages was confirmed by CD68 coexpression. It is well known that NPC is characterized by harboring EBV genes in the tumor cells and an intense infiltration of leukocytes in the stroma. These infiltrating cells are mainly composed of T lymphocytes and macrophages. The mechanism and role of this intense infiltration has been studied extensively (27). A strong association of macrophage infiltration with angiogenesis and prognosis in invasive breast cancer has been reported (28). Recently, Leek *et al.* (29) described the relation of HIF-2α expression in tissue-infiltrating macrophages to tumor angiogenesis and the oxidative thymidine phosphorylase pathway in human breast cancer. Our observation provides further support to the recognized contribution to tumor angiogenesis coming from the tumor-associated stromal microenvironment.

Other investigators frequently observed significant association between tumor necrosis and CA IX expression (17, 24, 30). We did observe that CA IX expression followed a perinecrotic pattern of distribution. However, necrosis was an infrequent finding in our biopsy specimens, probably reflecting the fact that the endoscopists often avoid taking a biopsy from the necrotic area of a tumor.

Recently, there have been several studies on the expression of HIF-1α, HIF-2α, and CA IX in a number of common human cancers. HIF-1α and HIF-2α were found to be overexpressed in most human cancers and their metastases (21, 25). HIF-1α overexpression was shown to be a marker of unfavorable prognosis in early-stage cervical cancer (31). HIF-1α expression was shown to predict resistance to photodynamic therapy in esophageal cancer (32) and was also found to be a novel predictive and prognostic parameter in oropharyngeal cancer treated by radiotherapy (33). In ovarian cancer, the overexpression of HIF-1α in combination with P53 indicated a dismal prognosis (34). Overexpression of HIF-1α indicated a shorter overall survival in oligodendrogliomas (35). In non-small cell lung cancer, HIF-2α expression was shown to be an independent prognosticator of poor outcome (36). However, another study in non-small cell lung cancer found that HIF-1-positive tumors had significantly longer median survival (37).

CA IX overexpression has also been shown in a number of common human tumors (26). In breast cancer, CA IX expression was associated with a worse relapse-free survival and overall survival (30). In cervical cancer, CA IX expression was shown to correlate with tumor hypoxia as measured by direct needle electrode, supporting its role as an intrinsic marker of tumor hypoxia, and was associated with poor prognosis (19). In non-small cell lung cancer, CA IX expression was shown to be a significantly poor prognosticator independent of angiogenesis (38). CA IX was also shown to relate to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy (39).

In head and neck cancer, VEGF overexpression was shown to be associated with poor disease-free and overall survival (40), a marker of tumor invasion and metastases (41). In NPC, expression of VEGF was found to have significant association with angiogenesis and lymph node metastases (42), as well as distant metastases (43).

NPC is distinct from other head and neck cancers in that it is highly sensitive to radiation and chemotherapy. In our study cohort of locally advanced nonmetastatic NPCs, HIF-1α expression was only associated with a borderline significant trend of poor overall survival, whereas no significant association with survival outcome was found for HIF-2α, CA IX, or VEGF. The

overall treatment result in this cohort was excellent in that all patients achieved complete remission after their primary treatment, despite their initial locally advanced stage. After a median follow up of 4.13 years, the median survival was still not reached yet. Because of the relatively small numbers of clinical events that occurred at the time of this analysis, we cannot exclude the potential prognostic significance of HIF-2α, CA IX, and VEGF in NPC, which have been reported by other authors in other tumor types. Another limitation of our study is that we used small biopsy tissue to determine the expression of the hypoxia markers, hoping it would reflect the hypoxia status of the whole tumor. Although this may be far from satisfactory, in NPC this is usually the only pathological material available to the clinician to base their treatment decision on.

Hypoxia is known to induce HIF-1α expression, which then up-regulates downstream genes *CA IX* and *VEGF* (16–18). HIF-1α and CA IX has been suggested to be endogenous markers of tumor hypoxia (19, 20, 44). We therefore proposed to define a model of tumor hypoxia and angiogenic profile based on the coexpression of HIF-1α, CA IX, and VEGF. In fact, our model predicted that tumors with a hypoxic profile (high expression of both HIF-1α and CA IX) were associated with a significantly worse PFS. Tumors with both hypoxic and angiogenic profile (high HIF-1α, CA IX, and VEGF expression) were associated with a significantly worse PFS, and this remained significant in multivariate analysis.

Tumor hypoxia is increasingly being recognized as an important therapeutic target (45). A whole panel of bioreductive drugs targeting at tumor hypoxia have been developed, and some have entered into Phase I/II/III clinical trials with promising preliminary results (46). Because we have established that HIF-1α, CA IX, and VEGF are expressed in the majority of NPCs, this represents a potential new therapeutic target for further development.

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