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Hypoxia-Inducible Factor-1α Expression Predicts a Poor Response to Primary Chemoendocrine Therapy and Disease-Free Survival in Primary Human Breast Cancer

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Abstract

Purpose: To investigate the relationship of hypoxia-inducible factor-1α (HIF-1α) tumor expression in predicting the response to epirubicin and disease-free survival (DFS) in patients with breast cancer enrolled in a single institution trial of primary anthracycline and tamoxifen therapy.

Experimental Design: The expression of HIF-1α was assessed by immunohistochemistry in 187 patients with T_{2-4} N_{0-1} breast cancer enrolled in a randomized trial comparing four cycles of single agent epirubicin versus epirubicin + tamoxifen as primary systemic treatment. All patients postoperatively received four cycles of the four weekly i.v. CMF regimen (cyclophosphamide, methotrexate, and 5-fluorouracil). Patients with estrogen receptor (ER)-positive primary tumors also underwent 5 years of treatment with adjuvant tamoxifen. Carbonic anhydrase IX (CAIX) was also scored as a marker of HIF activity.

Results: Overall response to therapy progressively decreased with increasing tumor HIF-1α ($P < 0.05$), and HIF-1α was an independent predictor of response ($P < 0.048$). HIF-1α expression was also associated with a significantly shorter DFS ($P < 0.02$) in all patients and in ER-positive but not in ER-negative patients. Furthermore, CAIX positivity conferred a significantly shorter DFS ($P = 0.02$) compared with CAIX-negative tumors in patients with HIF-1α-negative tumors.

Conclusions: HIF-1α expression in patients with breast cancer is a marker of poor therapy response and outcome, especially in ER-positive patients. The combination of two hypoxia markers has greater utility than assessing just one, and patients with hypoxia markers in their tumors may be suitable for administration of drugs that reduce HIF-1α expression and increase oxygen delivery to the tumor bed before starting neoadjuvant therapies.

Tumor growth and metastasis is dependent on the generation of a neovasculature. However, newly formed vessels function poorly in supplying oxygen and nutrient requirements in many tumors. Hypoxia, the pathophysiologic consequence of the structurally and functionally disturbed microcirculation (1), is therefore a common feature in solid tumors. Tumors respond to cellular oxygen deprivation using the ubiquitous family of transcription factors known as hypoxia-inducible factors (HIF; ref. 2). Under normal oxygen tension, HIF-1α is hydroxylated by specific prolyl hydroxylases, leading to recognition and binding by the von Hippel-Lindau protein, and targeting for degradation through the proteasome. In conditions of hypoxia, molecular oxygen is not available for hydroxylase activity, which leads to HIF-1α protein stabilization and translocation to the nucleus where it binds to aryl hydrocarbon nuclear translocators resulting in the activation of several gene pathways involved in angiogenesis, glycolysis, erythropoiesis, and apoptosis (see ref. 3). Overexpression of HIF-1α protein has been identified in many tumor types (4), with high levels influencing the growth rate and metastatic potential of these cancers. In breast cancers, the frequency of HIF-1α-positive cells increases in parallel with increasing pathologic stage and is associated with a poor prognosis (5–7). Furthermore, up-regulation of the hypoxia pathway by HIF has not only been shown to confer an aggressive phenotype, but also contributes to resistance to radiotherapy and chemotherapy.

Both radiotherapy and chemotherapy improve patient survival, with response dependent on tumor and patient

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Table 1. Patient characteristics

Total number	187
Randomization	
EPI	90 (48.1%)
EPI-TAM	97 (51.9%)
ER−	39 (20.1%)
ER+	147 (79.0%)
PgR−	96 (51.6%)
PgR+	90 (48.4%)
CAIX−	125 (75.3%)
CAIX+	41 (24.7%)
HIF-1α pretherapy	
0	33 (19.3%)
1	100 (58.5%)
2	38 (22.2%)
HIF-1α posttherapy	
0	17 (12.7%)
1	76 (56.7%)
2	41 (30.6%)
T_2	144 (77.0%)
T_{3-4}	43 (23.0%)
N_0	106 (56.7%)
N_1	81 (43.3%)
Grade 2	49 (26.6%)
Grade 3	135 (73.4%)
Missing	3
p53−	93 (50%)
p53+	93 (50.0%)
Missing	1
c-erbB2−	128 (74.9%)
c-erbB2+	43 (25.1%)
bcl2−	51 (27.4%)
bcl2+	135 (72.6%)
Clinical response	
Complete response	33 (17.7%)
Partial response	111 (59.7%)
No response	42 (22.6%)
Missing	1

characteristics. Predictive factors are used to forecast such response to a particular therapy (8). Each therapy should be evaluated independently in patient cohorts defined by the predictive factor (9). This can be optimally done in a prospective randomized clinical trial with primary chemotherapy being the optimal setting to study new biological markers in relation to the predictive information they provide. In addition, tumor biopsy specimens obtained in matched pair cases at diagnosis and definitive surgery provides valuable information on the interaction between biological markers and treatment. We have used an immunohistochemical approach to evaluate the putative hypoxia markers HIF-1α and carbonic anhydrase IX (CAIX) expression in a series of breast cancer specimens obtained before and after primary anthracycline and tamoxifen therapy. Our aims were (*a*) to test whether HIF-1α predicts response to treatment, (*b*) to assess whether HIF-1α predicts disease-free survival (DFS), and (*c*) whether using additional hypoxic markers helps define the hypoxic population.

Patients and Methods

Patients. Patients with T_{2-4} N_{0-1} breast cancer were recruited in a randomized trial comparing single agent epirubicin (EPI arm) versus epirubicin plus tamoxifen (EPI-TAM arm) as the primary systemic treatment (10). Patients were accrued from January 1997 to December 2001. The study was approved by the Institutional Investigations Committee. All patients gave written informed consent to the diagnostic procedures, the proposed treatment, and the biological evaluations. Two-hundred and eleven patients were enrolled, 105 were randomized to receive epirubicin alone, and 106 were randomized to receive epirubicin plus tamoxifen. On first presentation, an incision biopsy was done on each patient and a small tissue sample (0.5-0.8 cm) was removed. Chemotherapy was started within 2 days of diagnosis. Patients in the EPI arm received 60 mg/m^2 of epirubicin (Farmorubicina, Pharmacia, Milan, Italy) by slow i.v. push on days 1 and 2; whereas patients on the EPI-TAM arm received 60 mg/m^2 of epirubicin by slow i.v. push on days 1 and 2 and 30 mg of tamoxifen (Kessar, Pharmacia) daily. Epirubicin injections were repeated every 21 days for three or four cycles before definitive surgery, whereas tamoxifen was given continuously until definitive surgery. All patients postoperatively received four cycles of the CMF regimen [i.v. cyclophosphamide (600 mg/m^2), i.v. methotrexate (40 mg/m^2), and i.v. 5-fluorouracil (600 mg/m^2) on days 1 and 8, every 28 days; ref. 11]. Patients with estrogen receptor (ER)–positive primary tumor in both treatment arms received tamoxifen (20 mg, i.e., lower than the primary dose) starting after surgery, up to progression or for a maximum of 5 years. The median follow up of patients was 53 months (August 2004; range, 13-95).

Treatment evaluation. Each month, the size of the primary tumor and the size of the axillary lymph nodes, when appreciable, were measured by the same clinician using a caliper. Response was assessed before definitive surgery by the clinical measurement of the changes in the product of the two largest diameters recorded in two successive evaluations. According to WHO criteria, tumor progression was defined as an increase of at least 25% in tumor size; stable disease was defined as an increase of <25%, or a reduction of <50%; partial response was defined as a tumor shrinkage >50%; and complete response was defined as the complete disappearance of all clinical signs of disease. Pathologic complete response was defined as the absence of neoplastic cells in the breast and in the axillary lymph nodes. Surgery was planned after full clinical reassessment. Quadrantectomy or modified radical mastectomy were done when indicated in association with full axillary node dissection. All patients subjected to quadrantectomy underwent irradiation of the residual breast (60 Gy delivered over 6 weeks).

Histopathologic grade and immunohistochemistry. Tumor grade was evaluated using the Nottingham prognostic index (12). Immunohistochemical evaluation was done on paraffin-embedded tumor samples obtained at diagnosis and at definitive surgery. Bcl2, p53, ER, progesterone receptor (PgR), and Ki67 staining were done at the Pathology Unit of the Azienda Ospedaliera Istituti Ospitalieri of Cremona (Italy). The immunohistochemical method used in Cremona for routine markers is fully described elsewhere (13). Immunohistochemistry for HIF-1α and CAIX was done on 5 μm sections of tissue microarrays containing two 1-mm tumor cores taken from selected morphologically representative tumor regions of each paraffin-embedded breast tumor from both the initial diagnostic incisional biopsy and from tumor remaining at definitive surgery. Quality control was assessed on each block by H&E staining. HIF-1α was detected using the ESEE 122 (IgG_1 monoclonal antibody; dilution, 1:40) monoclonal antibody and CAIX with murine monoclonal antibody M75 (a kind gift from S. Pastorekova, Center of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic) at a dilution of 1:50 for 30 minutes (14). These were stained as previously reported by Talks et al. (15), and the

Envision HRP kit (Dako, Glostrup, Denmark) was used for subsequent visualization. Slides were counterstained with hematoxylin and mounted. Immunostaining for HIF-1α and CAIX were quantified in carcinoma cells by semiquantitative scoring as previously described (15, 16). Pathologists were blinded to patient outcome and whether the samples they examined were obtained from incisional biopsy or definitive surgery. Briefly, HIF-1α was scored as 0 (no staining), 1 (weak staining), or 2 (strong staining). Tumors were considered positive for HIF-1α and CAIX in survival analyses when any staining was present.

Statistical methods. χ^2 Test, χ^2 test for trend, and Fisher exact test were used when indicated to perform comparisons of proportions. Kruskal-Wallis ANOVA was done to compare continuous variables. DFS and overall survival were calculated from randomization to the occurrence of disease relapse or disease-related death. Patients were censored if they were free from recurrence and alive at the last follow-up period. The DFS and overall survival curves were estimated using the Kaplan-Meier method. Unadjusted differences in these estimates were assessed with the log-rank test. Multivariate logistic regression was used to identify covariates independently associated with disease response. The Cox proportional hazards model was used to assess, in multivariate analyses, the independent predictive role of clinicopathologic factors and the treatment administered for disease recurrence. All variables included in multivariate analyses were dichotomized variables with the exception of Ki67. This latter variable had a left-skewed distribution and was modeled using log-transformation. The stepwise backward procedure based on the likelihood ratio was employed in both multivariate analyses. All P values reported were two sided; $P < 0.05$ was considered statistically significant. Statistical analyses were done using Statistica for Windows (Tulsa, OK) and SPSS for Windows software packages.

Results

Patient characteristics. One hundred and eighty-seven of the 211 (88.6%) patients prospectively enrolled in the trial were evaluable for HIF-1α. For the remaining 24 patients, insufficient material was available. Patient characteristics are shown in Table 1. Ninety patients (48%) were randomized to the EPI arm and 97 (52%) patients were randomized to the EPI-TAM arm. HIF-1α was evaluated at baseline excision in 171 (91%) tumors, 134 (72%) in the main tumor resection,

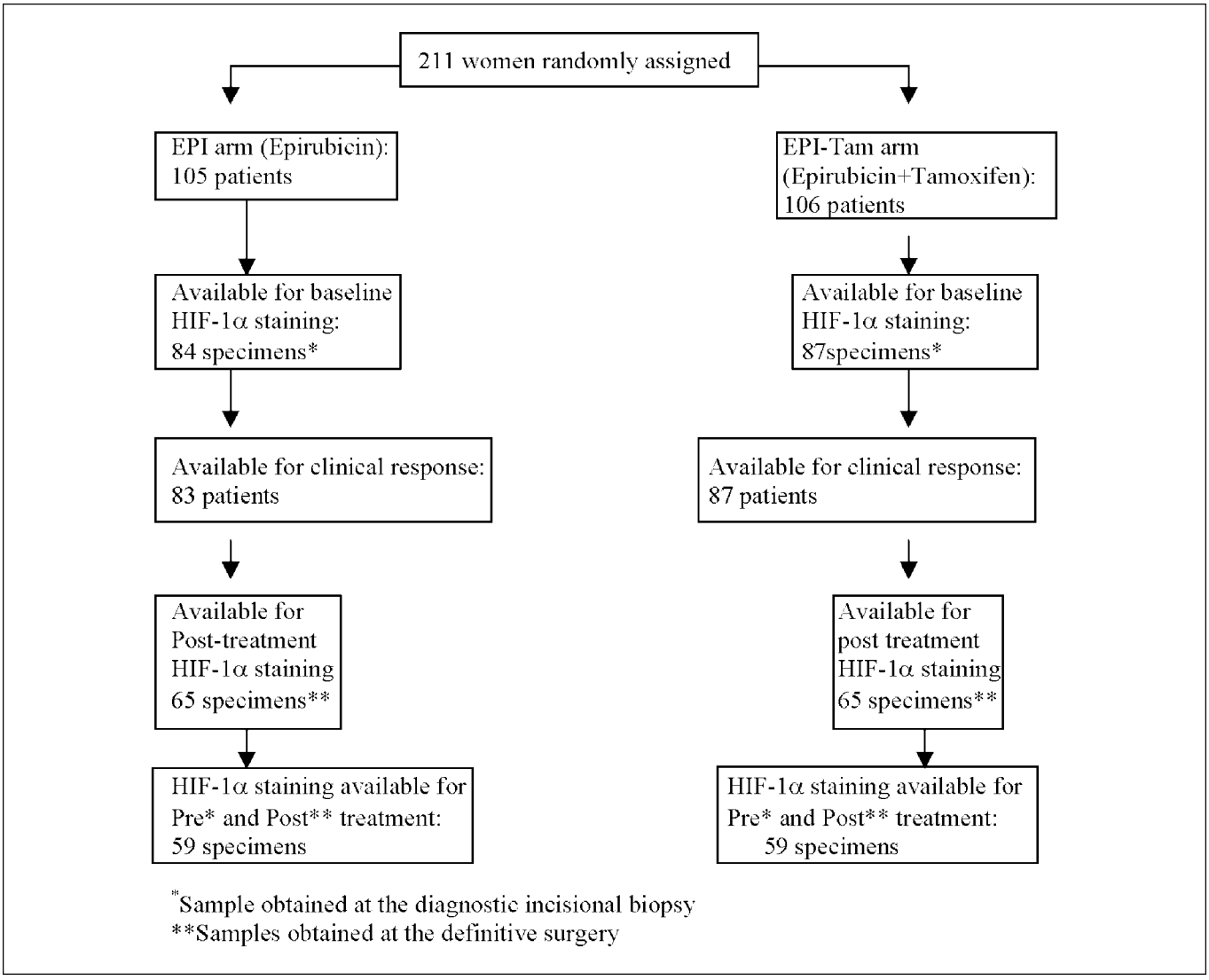

Fig. 1. CONSORT diagram.

Table 2. Distribution of prognostic variables and CAIX expression according to HIF-1α expression

Grading	0	1	2	*P*	
				Trend	0 and 1 versus 2
Grade 2	10 of 33 (30.3%)	28 of 99 (28.3%)	7 of 36 (19.4%)		
Grade 3	23 of 33 (69.7%)	71 of 99 (71.7%)	29 of 36 (80.5%)	0.30	0.26
p53	16 of 33 (48.5%)	49 of 100 (49.0%)	19 of 37 (51.3%)	0.81	0.78
c-erb-B2	8 of 33 (24.2%)	23 of 100 (23.0%)	12 of 38 (31.6%)	0.45	0.30
bcl2	28 of 33 (84.8%)	74 of 100 (74.0%)	24 of 37 (64.8%)	0.06	0.14
ER	27 of 33 (81.8%)	81 of 99 (81.8%)	29 of 38 (66.3)	0.75	0.44
PgR	19 of 33 (57.6%)	49 of 99 (49.5%)	15 of 38 (39.5%)	0.12	0.19
T_2	27 of 33 (81.8%)	74 of 100 (74.0%)	30 of 38 (78.9%)	0.81	0.70
T_3	6 of 33 (18.2%)	26 of 100 (26.0%)	8 of 38 (21.1%)		
Node positive	16 of 33 (48.5%)	40 of 100 (40.0%)	20 of 38 (52.6%)	0.67	0.25
Ki67	21.6 (14.7-28.4)	21.5 (17.9-25.1)	24.8 (17.4-32.1)	0.66	0.66
CAIX	8 of 29 (27.6%)	20 of 36 (20.8%)	13 of 36 (36.1%)	0.36	0.09

and 118 (63%) before and after treatment, whereas 16 (9%) patients had HIF-1α assessed in the residual tumor histology only. Positive tumor immunostaining was detected in 138 (80.7%) tumor samples collected before treatment and in 117 (87.3%) tumor samples collected afterwards (Fig. 1). CAIX was expressed in 41 of 166 (24.7%) pretreatment biopsies (Table 1).

Relationship between baseline HIF-1α expression and clinicopathologic variables. The relationship between HIF-1α expression and clinicopathologic variables was assessed by both χ^2 for trend and χ^2, the latter test was done to compare patients with strong tumor expression of HIF-1α compared with those with low or no expression. HIF-1α was scored as 0 (no staining), 1 (weak staining), or 2 (strong staining). There was no correlation between HIF-1α and c-erb-B2, T status, N status, grade, p53, bcl2, or ER in a univariate analysis of expression of preneoadjuvant tumors ($P > 0.05$; Table 2).

Effect of treatment on HIF-1α expression. In the 118 patients with HIF-1α assessed in matched samples before and after treatment, HIF-1α positivity was found in 98 baseline tumor samples (83.1%) and in 106 residual tumor samples after chemotherapy (89.8%). When considering individual variation, HIF-1α status changed from positive to negative in seven patients (5.9%), one (1.7%) randomized in the EPI arm and six (10.2%) in the EPI-TAM arm, respectively. The opposite change (negative to positive) occurred in 15 cases (12.7%), 8 (13.6%) randomized in the EPI arm and 7 (11.9%) in the EPI-TAM arm, respectively (Fig. 2A). As a consequence of the different changes between treatment arms, HIF-1α positivity at postchemotherapy histology was significantly lower in the EPI-TAM arm (83.1%) as opposed to the EPI arm (96.4%; Fisher χ^2 test; $P < 0.03$; Fig. 2B).

HIF-1α expression and response to treatment. Among the 171 patients with HIF-1α assessed at baseline, one patient refused to continue the treatment after the first cycle and was not assessable for response. One hundred and thirty-three out of 170 assessable cases (78.2%) showed a clinical response (complete + partial), 31 (18.2%) cases showed a complete response, and 102 showed a partial response (60.0%). At postchemotherapy residual histology, five patients (2.9%) had a pathologic complete response. According to HIF-1α expression, overall response progressively decreased with an increase in HIF-1α expression score ($P < 0.05$; Table 3). Stratifying patients according to treatment arm, the inverse correlation between HIF-1 expression and disease response was evident in EPI but not in EPI-TAM patients. The five pathologically complete responses were observed in patients with negative or weak HIF-1α expression. In a multivariate analysis using a logistic regression model using 0, 1, and 2 as in univariate analysis, HIF-1α expression was confirmed to be a significant independent variable predicting clinical response

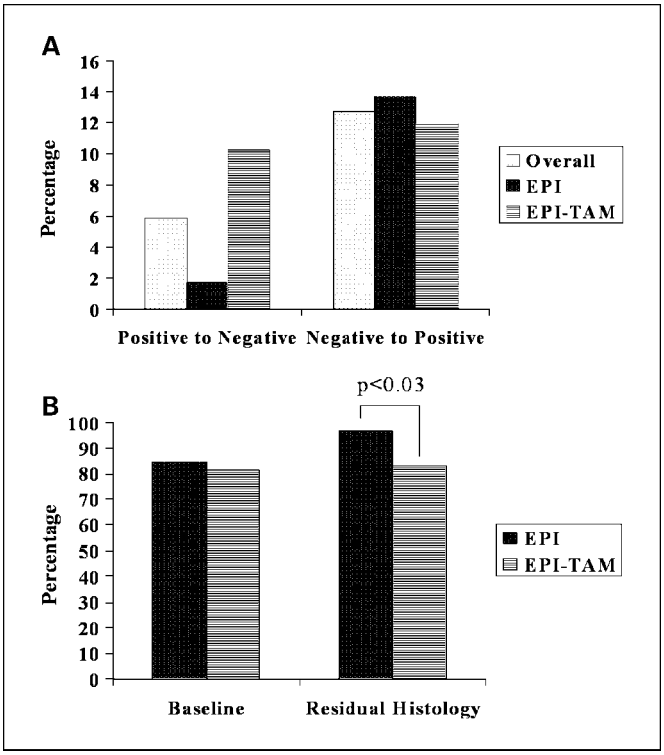

Fig. 2. *A,* HIF-1α individual variation from positive to negative and the opposite change (negative to positive) overall and according to the randomization. *B,* HIF-1α expression changes between treatment arms.

Table 3. Disease response according to baseline HIF-1α expression

	0	1	2	P
Clinical response (complete + partial)				
All patients under Clinical Response	18 of 33 (84.8%)	80 of 99 (80.8%)	25 of 38 (65.8%)	<0.05*
EPI	12 of 14 (85.7%)	38 of 49 (77.6%)	11 of 20 (55.0%)	<0.04*
EPI-TAM	16 of 19 (84.2%)	42 of 50 (84.0%)	14 of 18 (77.8%)	0.61*
Pathologic response				
Pathologic complete response	1 of 33 (3.0%)	4 of 99 (4.0%)	0 of 38 (0)	0.45

*χ^2 Test for trend.

(odds ratio, 0.546; 95% confidence interval, 0.299-0.995; P = 0.048) after adjusting for T stage, N status, steroid hormone receptor status, c-erb2, bcl2, p53, and Ki67 expression (Table 4).

HIF-1α expression and disease outcome. Forty-four out of 187 patients relapsed (23.5%) and 22 (11.7%) died of disease. HIF-1α expression was associated with a statistically significant shorter DFS (P = 0.02; Fig. 3), whereas overall survival was not affected (data not shown). Stratifying patients according to ER status, showed HIF-1α to be a significant predictor of shorter DFS in ER-positive (Fig. 4) but not in ER-negative patients, the latter probably reflecting low numbers in each subgroup.

In multivariate analysis, HIF-1α expression failed to be independently associated with DFS after adjusting for T stage, N status, steroid hormone receptor status, c-erb2, bcl2, p53, and Ki67 (hazard ratio, 2.56; 95% confidence interval, 0.77-8.50; P = 0.12). However, because HIF-1α has a half-life that is measured in minutes, we also assessed whether the hypoxia marker, CAIX, which has a half-life of 38 hours, was associated with a worse prognosis. We therefore stratified HIF-1α-negative patients by CAIX. This showed that those patients whose tumors expressed CAIX had a significantly shorter DFS (P = 0.02) than patients with tumors showing no CAIX expression (Fig. 5). A similar but nonsignificant trend was also observed in HIF-1α-positive tumors in which CAIX positivity was associated with a shorter DFS (P = 0.09).

Discussion

The search for associations between biological factors and treatment response is important not only to distinguish patients that will derive benefit from certain regimens from those who will not, but also to identify novel targets for specific therapeutics. This study has focused on the evaluation of the key hypoxia mediator, HIF-1α, as a predictive and prognostic factor.

Although HIF-1α expression may also be influenced by other pathways (17), a significant correlation between oxygen tension and HIF-1α has been reported in cervical cancer (18), suggesting that HIF-1α might be used as a surrogate for tumor hypoxia. HIF-1α expression is associated with a reduced survival in a variety of human cancers including uterine cervix (19), ovary (20), esophagus (21), lung (22), and breast (6, 23) and may also influence resistance to therapy in several cancer types (see ref. 24). Here, we show for the first time in human breast cancer that HIF-1α expression is also a predictive marker of chemotherapy failure. We observed that pretreatment levels of HIF-1α showed a significant inverse correlation with disease response; tumors with a high expression of HIF-1α being less likely to respond to chemotherapy. It is interesting that only

Table 4. Multivariate logistic regression analysis of independent predictive factors for clinical response

	Hazard ratio (95% confidence interval)	P
HIF-1α	0.546 (0.299-0.995)	0.048
Ki67	1.249 (0.273-5.718)	0.774
N status	0.842 (0.372-1.904)	0.680
p53	0.861 (0.386-1.921)	0.714
bcl2	1.162 (0.458-2.951)	0.752
ER	0.667 (0.179-2.477)	0.542
PgR	1.288 (0.529-3.140)	0.578
c-erbB2	0.646 (0.272-1.533)	0.326
Tstatus	0.602 (0.259-1.399)	0.245
Treat	1.655 (0.776-3.530)	0.190
EPI versus EPI-TAM		
Grade	1.829 (0.784-4.262)	0.167

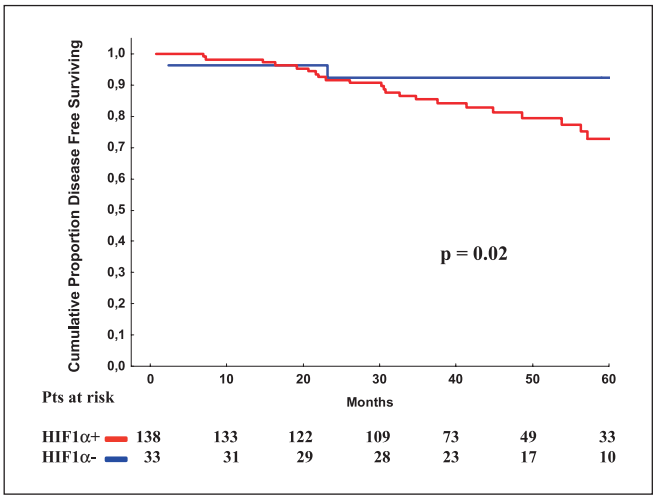

Fig. 3. Kaplan-Meier curves of disease-free survival of patients stratified by HIF-1α expression.

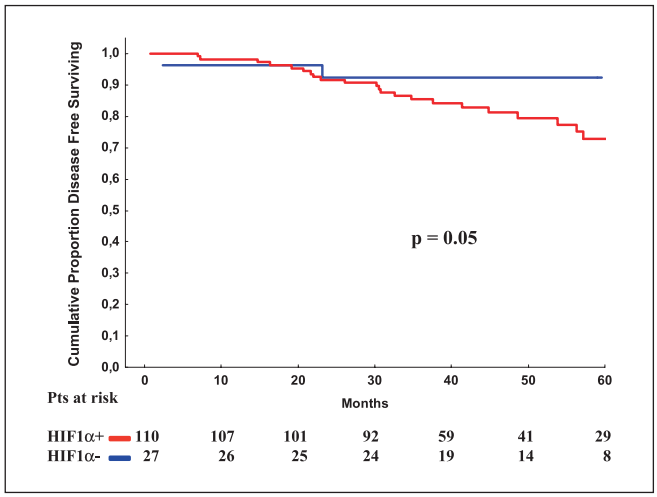

Fig. 4. Kaplan-Meier curves of disease-free survival of patients with ER-positive tumors stratified by HIF-1α expression.

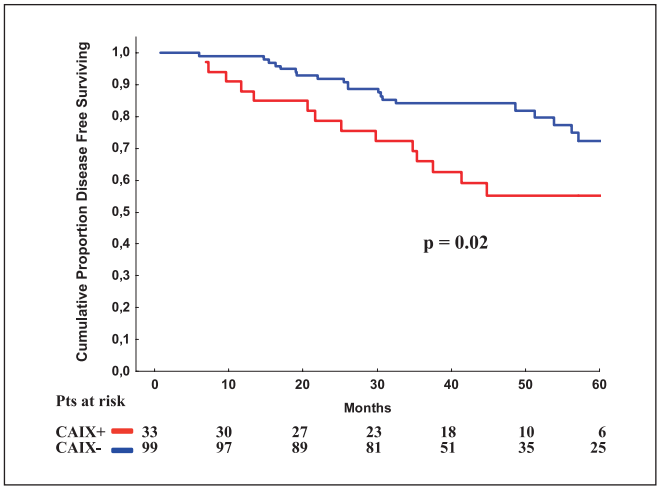

Fig. 5. Kaplan-Meier curves of disease-free survival of patients with HIF-1α-negative tumors stratified by CAIX expression.

patients with absent or weak HIF-1α expression showed a pathologic complete response.

This data also confirms *in vitro* and *in vivo* data which suggests that hypoxia reduces the effects of certain cytotoxic agents, particularly anthracyclines (25, 26). This is in support of our previous findings of the negative effect of low hemoglobin on radiation and antineoplastic agents in patients with breast cancer (1, 25, 27) and implies that the concurrent administration of erythropoietin to maintain oxygen delivery to the tumor bed would increase drug efficacy. Alternatively, the addition of taxanes concomitantly or sequentially to anthracycline-based regimens may also increase tumor oxygenation (28).

An increase in HIF-1α expression was more frequently observed in patients treated with epirubicin alone than with the chemoendocrine combination therapy, suggesting that this agent may at least partly be responsible for HIF-1α induction. However, because anthracycline therapy reduces blood flow (29), this effect might also account for HIF-1α induction and the stimulation of vascular endothelial growth factor noted with anthracycline therapy (30). However, antiestrogens such as tamoxifen have also been shown to inhibit angiogenesis in both an estrogen-dependent and -independent manner. Although this may also be expected to enhance the hypoxic fraction of the tumor, the concomitant tumor cell apoptosis from the combination treatment could result in a reduction of interstitial pressure (31), abrogating the effect. The potential mechanisms for HIF-mediated drug resistance include altering the apoptotic pathway (26), up-regulating the multidrug resistance transporter P-glycoprotein (32), in addition to the poor drug delivery in functionally deficient vessels.

Our data shows that HIF-1α not only selects the patients with a higher risk of relapse, but also identifies the ER-positive patients with a poor outcome that is similar to that of ER-negative patients. These findings, together with the observation of HIF-1α being an indicator of poor prognosis in node-negative and -positive breast cancers (6, 23), suggests that whatever the biological background, hypoxia is an essential element in the selection of more aggressive phenotype being associated with both chemoresistance and endocrine resistance. Indeed, because HIF-1α protein has a half-life that is measured in minutes (33), we also wished to determine whether the presence of chronic, in addition to recent, hypoxia conferred a poor prognosis. Therefore, we also stratified HIF-1α-negative patients by CAIX expression. CAIX is a HIF-induced protein, which has a half-life of 38 hours (34), longer than that of HIF-1α (and therefore accounting for the absence of association between HIF-1α and CAIX). It is interesting to note that the presence of tumor CAIX was significantly associated with a worse DFS in these patients compared with CAIX-negative tumors. This enzyme contributes to an acidic extracellular environment, which is known to reduce anthracycline uptake.

In conclusion, the measurement of HIF-1α in breast cancer may be of use to prospectively stratify patients in trials of neoadjuvant therapy using drugs which are able to increase oxygen delivery to the tumor or inhibit HIF, e.g., bortezomib.

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