

Overexpression of Hypoxia-inducible Factor 1 α Is Associated with an Unfavorable Prognosis in Lymph Node-positive Breast Cancer

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ABSTRACT

Purpose: Hypoxia-inducible factor (HIF)-1 α is a transcription factor that supports the adaptation of human cancer cells to hypoxia and is involved in various pathways supporting tumor growth and progression. The aim of this study was to determine the prognostic influence of HIF-1 α expression in patients with advanced-stage breast cancer, evident by positive lymph nodes.

Experimental Design: Expression of HIF-1 α was determined immunohistochemically in 206 patients with lymph node-positive breast cancer. Furthermore, the interrelationship of HIF-1 α with p53 and HER-2 protein expression, estrogen receptor density, and survival was analyzed. Colocalization of p53 and HIF-1 α proteins was analyzed by confocal laser scanning microscopy.

Results: Strong nuclear expression of HIF-1 α by invasive cancer cells was found in 48 patients (23.3%), moderate expression was found in 74 patients (35.9%), and weak expression was found in 35 patients (17%); no expression was observed in 49 patients (23.8%). HIF-1 α protein overexpression was associated with significantly shorter overall and disease-free survival time ($P = 0.003$ and $P = 0.001$, respectively; Cox regression analysis). No correlation of HIF-1 α and HER-2 expression or estrogen receptor density was observed.

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Conclusions: This study shows that HIF-1 α is an independent prognostic factor for an unfavorable prognosis in patients with lymph node-positive breast cancer. Our results indicate that patients with advanced-stage breast cancers might profit from future therapies targeting HIF-1 α .

INTRODUCTION

Neoangiogenesis is considered essential for tumor growth and the development of metastases, as well as for the progression of preinvasive precursor lesions to invasive cancer. Because cancer cell proliferation may outpace the rate of angiogenesis, thus causing hypoxia (1), the adaptation of tumor cells to tissue hypoxia is of central importance for tumor progression (2).

The mechanisms leading to neoangiogenesis and in particular to the adaptation of tumor cells to hypoxia are still poorly understood. One of the key factors regulating cellular O₂ homeostasis is the transcription factor HIF³⁻¹ (3, 4). HIF-1 is a heterodimeric complex composed of the two bHLH-PAS subunits, HIF-1 α and HIF-1 β (Ref. 5; PAS refers to the first proteins in which this motif was identified, *i.e.*, PER refers to the protein product of the *Drosophila period* gene, ARNT refers to the aryl hydrocarbon receptor nuclear translocator, and SIM refers to the protein product of the *Drosophila single-minded* gene). The bHLH domain mediates dimerization and DNA binding in many transcription factors. PAS is an additional dimerization motif. Whereas HIF-1 β is a common subunit of multiple bHLH proteins, HIF-1 α is the unique, O₂-regulated subunit that determines HIF-1 activity (4). HIF-1 α influences a number of genes that, in part, play a role in tumor progression including erythropoietin, transferrin, endothelin-1, inducible nitric oxide synthetase, hemoxygenase 1, VEGF, insulin-like growth factor-2, insulin-like growth factor-binding proteins 2 and 3, and 13 different glucose transporters and glycolytic enzymes (3).

Induction of HIF-1 α expression appears to be a critical step in the hypoxic response. It occurs via increased mRNA expression, protein stabilization, nuclear localization, and augmented activity of its transcriptional activation domains (3). Nuclear accumulation of this protein can be detected immunohistochemically.

There is increasing evidence that HIF-1 α is one of the key factors in the progression of human malignant disease (6–9). Several studies revealed that HIF-1 α protein may be demonstrated in a variety of human cancers (7, 8). However, until now only few data exist on the impact of HIF-1 α expression on the

³ The abbreviations used are: HIF, hypoxia-inducible factor; bHLH, basic helix-loop-helix; VEGF, vascular endothelial growth factor; OS, overall survival; DFS, disease-free survival; DCIS, ductal carcinoma *in situ*.

prognosis of human malignant diseases (10–13). It appears that HIF-1 α has a dual function. On one hand, it stimulates angiogenesis via, *e.g.*, transactivation of the *VEGF* gene, thus supporting tumor growth (14). On the other hand, HIF-1 α may associate with p53 protein, thus increasing the stability of p53, ultimately leading to an increased apoptotic rate of (tumor) cells expressing HIF-1 α (15). Considering the results in patients with cervical cancer, it was, therefore, suggested that the negative prognostic significance of HIF-1 α may be particularly strong because p53 is inactivated by the human papillomavirus oncoprotein E6 in these patients (10). In this patient group, the tumor-suppressive functions of HIF-1 α may be, therefore, lost already in initial stages in the majority of cases through the influence of human papillomavirus infection and p53 protein inactivation. The function remaining is the angiogenic property enhancing tumor growth, thus explaining the unfavorable prognosis of patients overexpressing HIF-1 α protein. This notion was supported by a study of ovarian cancer, where the combination of p53 protein accumulation, which signifies a loss of function of p53 (16), with HIF-1 α protein overexpression was associated with the most rapid progression of the disease (11).

Until now, the effect of HIF-1 α expression on the prognosis of breast cancer, which is the most important female cancer, had not been studied. A recent work that suggested an influence of HIF-1 α expression on the progression of early-stage disease (DCIS) to invasive breast cancer (17) supported the notion that HIF-1 α protein overexpression may be of relevance in neoplastic breast disease.

The aim of this study was to determine the impact of HIF-1 α protein expression and its relation with p53 protein overexpression, which is highly associated with the presence of nonfunctional p53 protein (18), on the prognosis of patients with breast cancer showing axillary lymph node metastases at the time of surgery.

PATIENTS AND METHODS

Study Population. The study population consisted of 206 unselected cases of invasive breast cancer that were part of two prospective studies of the Austrian breast cancer study group (studies 2 and 4; Refs. 19, 20). All patients had lymph node-positive breast cancer with at least 10 lymph nodes isolated from the axillary fatty tissue.

Methods. The expression of HIF-1 α and p53 proteins was determined immunohistochemically in paraffin-embedded tumor specimens fixed in 4% buffered formalin. Histological slides, 4 μ m in thickness, were deparaffinized in xylol. Slides were heated in 0.01 M citrate buffer for 16 min in a microwave oven. After cooling for 20 min and washing in PBS, endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 min, followed by incubation with PBS containing 10% normal goat serum for 30 min. For immunohistochemical detection of HIF-1 α , specimens were incubated overnight at 4°C with a monoclonal anti-HIF-1 α antibody (clone monoclonal antibody H1 α 67, NB 100-105; Novus Biologicals, Littleton, CO; Refs. 7, 21) at a dilution of 1:60. Visualization of bound antibodies was performed with streptavidin-biotin-peroxidase complex technique (Super Sensitive kit; BioGenex, San Ramon, CA). As a chromogene, 3-amino-9-

ethylcarbazole (BioGenex) was used. Expression of p53 was investigated with monoclonal antibody DO-7 (DAKO, Glostrup, Denmark) using a standard protocol (22). Immunohistochemistry for HER-2/neu protein expression was performed using the HercepTest (DAKO), according to the manufacturer's instructions (23).

As a positive control for HIF-1 α expression, immunostaining was performed on a sample of cervical cancer tissue with known strong expression of HIF-1 α , which has also been used in a previous study (10). Appropriate human colon cancer tissue was used as a positive control for the study of p53. For a negative control, primary antibodies were replaced by appropriate mouse IgG. As additional negative control, immunostaining for HIF-1 α was also performed in five samples of normal cervical tissues known to be HIF-1 α negative.

Nuclear expression of HIF-1 α , representing the biologically active form of this transcription factor, was determined by assessing semiquantitatively the percentage of decorated tumor cells and the staining intensity (10). The percentage of positive cells was rated as follows: 0 points, cases with \leq 10% positive cells were rated as negative, regardless of staining intensity; 2 points, 11–50% positive cells; 3 points, 51–80% positive; and 4 points, >80% positive cells. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for percentage of positive cells and staining intensity were added, and specimens were attributed to four groups according to their overall score: negative expression, \leq 10% of cells stained positive, regardless of intensity; weak expression, 3 points; moderate expression, 4–5 points; and strong expression, 6–7 points. A specimen was considered as "positive" for p53 expression when >50% of tumor cells showed distinct nuclear staining, and the remainder was considered "negative" with regard to p53 expression (24).

Two independent observers (P. B. and G. O.) performed the analysis of immunohistochemistry. The mean values of results from both observers were used for all additional calculations. If differences of >30% between observers occurred (evident in <10% of cases), these slides were reinvestigated by both investigators on a multiheaded microscope. Estrogen receptor density was determined using the dextran charcoal method from snap-frozen tumor samples.

To further investigate a possible interaction between p53 and HIF-1 α proteins, multichannel confocal laser scanning microscopy using a LSM 510 (Zeiss, Oberkochen, Germany) was performed. The colocalization between p53 (antibody DO-7) and HIF-1 α protein (antibody NB 100-105) was assessed in five selected samples of p53-positive breast cancer specimens. Alexa Fluor 488- and 633-labeled secondary antibodies and propidium iodide for nuclear staining (all from Molecular Probes, Inc., Eugene, OR) were used according to a standard protocol.

Statistics. Spearman's coefficient of correlation, the Kruskal-Wallis test, and the Mann-Whitney test were used as appropriate. OS was defined from the day of surgery until death of the patient. Data on patients who had survived until the end of the observation period were censored at their last follow-up visit. Deaths from a cause other than breast cancer were considered censoring events. DFS was defined from the end

of primary therapy until first evidence of progression of the disease.

Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier (25). The Cox proportional hazards model was used for multivariate analysis. HIF-1 α score, HER-2 staining intensity, patient's age at time of diagnosis (≤ 50 versus > 50 years), menopausal status, histological grading, estrogen receptor density, and tumor stage were entered into Cox regression. For all tests, $P \leq 0.05$ was considered as significant. All P s given are the results of two-sided tests. This study by the Austrian Breast Cancer Study Group was performed after approval by a local human investigations committee.

RESULTS

Study Population. The mean age of the patients was 52.3 ± 10.4 years; 95 (46.1%) patients were postmenopausal, and 97 (47.1%) patients were premenopausal. In 14 patients, the menopausal status was not known (6.8%). Patients were treated surgically by lumpectomy or mastectomy. According to the International Union against Cancer criteria, stage 1 (tumor ≤ 2 cm) was found in 107 (51.9%) specimens and stage 2 (tumor > 2 cm but ≤ 5 cm) was found in 85 (41.3%) specimens; in 14 (6.8%) cases, the stage was unknown. Histological tumor grade, as determined by a modified Bloom and Richardson score (26), was grade I in 14 (6.8%), grade II in 111 (53.9%), and grade III in 81 (39.3%) specimens (determined by G. O.).

A combined adjuvant chemotherapy (on day 1, 20 mg/m² doxorubicin and 1 mg/m² vincristine, and on days 29 and 36, 300 mg/m² cyclophosphamide, 25 mg/m² methotrexate, and 600 mg/m² fluorouracil) in combination with tamoxifen was applied to 94 (45.6%) patients. Tamoxifen alone was administered to 78 (37.9%) subjects, and 34 (16.5%) patients did not receive any adjuvant therapy. Tamoxifen was administered at a dose of 2×10 mg p.o. daily for 2 years. The median follow-up time was 87 months (range, 9–170 months). During this observation period, 91 (44.2%) patients developed recurrent disease, and 73 (35.4%) patients died from breast cancer.

Immunohistochemistry. Normal breast tissue was generally negative for HIF-1 α expression, with the exception of some cases where normal breast tissue directly adjacent to formations of invasive cancer showed moderate nuclear expression. Adjacent formations of DCIS commonly expressed HIF-1 α as described previously (17).

Strong nuclear expression of HIF-1 α by invasive cancer cells was found in 48 (23.3%) patients (Fig. 1A), moderate expression was found in 74 patients (35.9%), and weak expression was found in 35 (17%) patients (Fig. 1B); no expression of HIF-1 α was observed in 49 (23.8%) patients. In general, strong nuclear staining intensity was also associated with strong cytoplasmic staining.

Forty-four patients were considered positive with regard to p53 expression. Twenty-five (12.1%) patients showed a combination of strong or moderate HIF-1 α expression with p53 overexpression. During observation times, 15 (60%) of these patients developed recurrent disease, and 13 (52%) died of breast cancer.

HER-2 expression was rated 0/1+ in 154 (74.8%) patients,

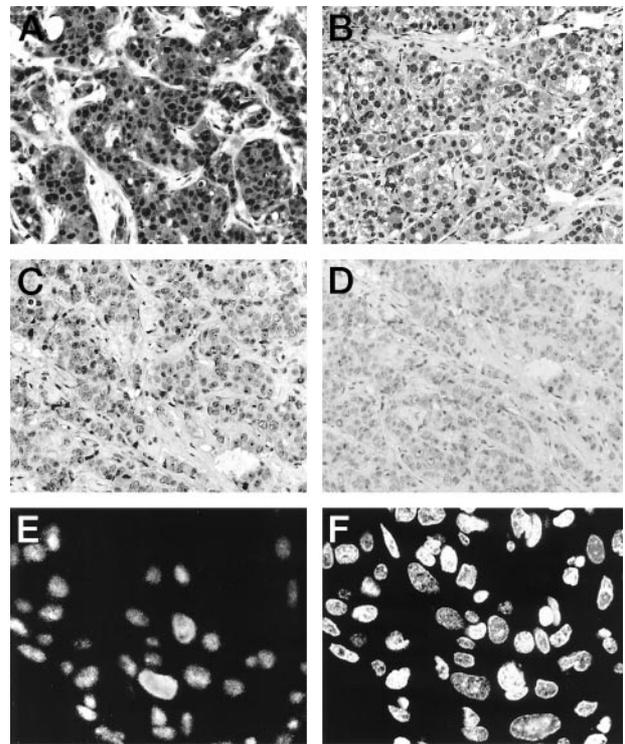


Fig. 1 A, specimen of invasive breast cancer with strong expression of HIF-1 α in a vast majority of tumor cells. Note also the strong cytoplasmic staining. Immunoperoxidase staining, $\times 200$. B, specimen of invasive breast cancer with moderate nuclear expression of HIF-1 α in tumor cells. Note the considerable weaker cytoplasmic staining compared with A. Immunoperoxidase staining, $\times 200$. C, specimen of invasive breast cancer with weak nuclear expression of HIF-1 α in a subset of tumor cells. Note the absence of cytoplasmic staining. Immunoperoxidase staining, $\times 200$. D, specimen of invasive breast cancer with absent expression of HIF-1 α in tumor cells. Immunoperoxidase staining, $\times 200$. E, HIF-1 α expression in a specimen of p53-positive breast cancer determined by confocal laser scanning microscopy. Immunofluorescence, $\times 800$. F, p53 expression in a specimen of p53-positive breast cancer determined by confocal laser scanning microscopy (same field of view as in E). Immunofluorescence, $\times 800$.

2+ in 28 (13.6%) patients, and 3+ in 24 (11.7%) patients. Mean estrogen receptor density was 91.9 ± 123.5 fmol/l. There was no correlation of HIF-1 α expression with histological grading ($P = 0.479$, Spearman's coefficient of correlation), estrogen receptor density ($P = 0.45$, Kruskal-Wallis test), p53 expression ($P = 0.185$, Mann-Whitney test), tumor stage (0.175, Mann-Whitney test), or HER-2 expression ($P = 0.447$, Kruskal-Wallis test).

Confocal Microscopy. Confocal microscopy analysis revealed that in p53-positive cancers, the vast majority of HIF-1 α -expressing cells also expressed p53 protein. Nevertheless, we also observed frequently single cells expressing HIF-1 α but not p53 (Fig. 1, E and F).

Survival Analysis. In univariate analysis, a significant influence of HIF-1 α expression on OS was found ($P = 0.0454$, log-rank test; Fig. 2A). The 5-year OS rate was 75.31% in patients with absent and low expression of HIF-1 α , 61.26% in patients with moderate expression of HIF-1 α , and 59.25% in

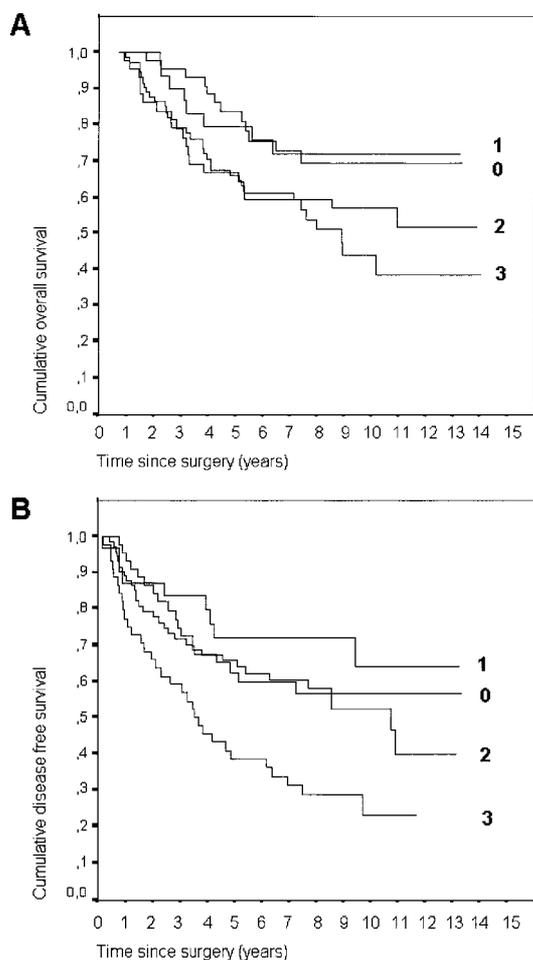


Fig. 2 A, cumulative overall survival in 206 patients with lymph node-positive breast cancer with strong expression (Line 3), moderate expression (Line 2), low expression (Line 1), and no expression (Line 0) of HIF-1 α ($P = 0.0454$, log-rank test). B, cumulative DFS in 206 patients with lymph node-positive breast cancer with strong expression (Line 3), moderate expression (Line 2), low expression (Line 1), and no expression (Line 0) of HIF-1 α ($P = 0.0008$, log-rank test).

patients with strong expression of HIF-1 α . In addition, a significant influence of histological grading ($P = 0.0073$) and tumor stage ($P = 0.0216$) on OS was found in univariate analysis. No influence on OS in univariate analysis was found for HER-2 expression (although a clear trend toward diminished prognosis in 3+ patients was observed), patient's age, p53 status, estrogen receptor density, and therapy regimen ($P > 0.05$). In multivariate analysis, HIF-1 α expression, tumor stage, and histological grading remained independent prognostic factors (Table 1).

At univariate analysis of DFS, HIF-1 α expression ($P = 0.0008$), histological grading ($P = 0.0003$), and HER-2 expression ($P = 0.0073$) showed a significant influence on prognosis (Fig. 2B), whereas the patient's age, p53 status, estrogen receptor density, tumor stage, and therapy regimen did not influence DFS ($P > 0.05$). In multivariate analysis of DFS, only HIF-1 α expression and histological grading remained independent prognostic factors (Table 1).

Table 1 Survival of patients with lymph node-positive breast cancer ($n = 206$; Cox regression)

	<i>P</i>	95% confidence interval	Relative risk
OS			
HIF-1 α expression	0.003	1.12–1.77	1.41
Histological grading	0.002	1.29–3.1	2
Patient's age	0.271		
Tumor stage	0.034	1.04–2.64	1.66
Estrogen receptor density	0.839		
HER-2 expression	0.85		
DFS			
HIF-1 α expression	0.001	1.14–1.72	1.4
Histological grading	<0.001	1.45–3.17	2.15
Patient's age	0.323		
Tumor stage	0.138		
Estrogen receptor density	0.565		
HER-2 expression	0.955		

In addition, patients were stratified into four groups according to HIF-1 α and p53 expression: (a) absent or weak HIF-1 α expression/absent p53 expression ($n = 65$); (b) absent or weak HIF-1 α expression/positive p53 expression ($n = 19$); (c) moderate or strong HIF-1 α expression/absent p53 expression ($n = 97$); and (d) moderate or strong HIF-1 α expression/positive p53 expression ($n = 25$). In univariate analysis, a significant difference in OS ($P = 0.0005$, log-rank test; Fig. 3A) and DFS ($P = 0.0078$, log-rank test) was observed between groups (Fig. 3B), which failed to reach significance in multivariate analysis ($P > 0.05$, Cox regression).

DISCUSSION

Expression of HIF-1 α has been demonstrated recently to be associated with a more aggressive phenotype of cancer cells and with impaired clinical outcome in a variety of human tumors, including cervical, ovarian, and oropharyngeal cancer and cerebral oligodendrogliomas (10–13). Because there were no data available on the prognostic relevance of HIF-1 α expression in human breast cancer, this study was undertaken. We were particularly interested in subjects with advanced-stage disease, because patients presenting with lymph node metastases at the time of surgery have a significantly impaired prognosis, compared with those with disease limited to the breast (27). Therefore, a better understanding of tumor biology is urgently required, especially in this group of patients, that would allow the development of new, more effective therapies.

Whereas the majority of tumor specimens showed an expression of HIF-1 α in varying density, normal breast tissue was shown to be HIF-1 α negative. The fact that in several cases normal breast parenchyma directly adjacent to invasive tumor formations expressed HIF-1 α might be explained by the occurrence of hypoxia in the vicinity of cancer cells initiating the expression of HIF-1 α in normal breast tissue as part of a physiological response.

This study demonstrates for the first time that HIF-1 α overexpression is an independent prognostic factor in advanced-stage breast cancer. In these groups, DFS as well as OS were significantly shorter as compared with cases showing no or

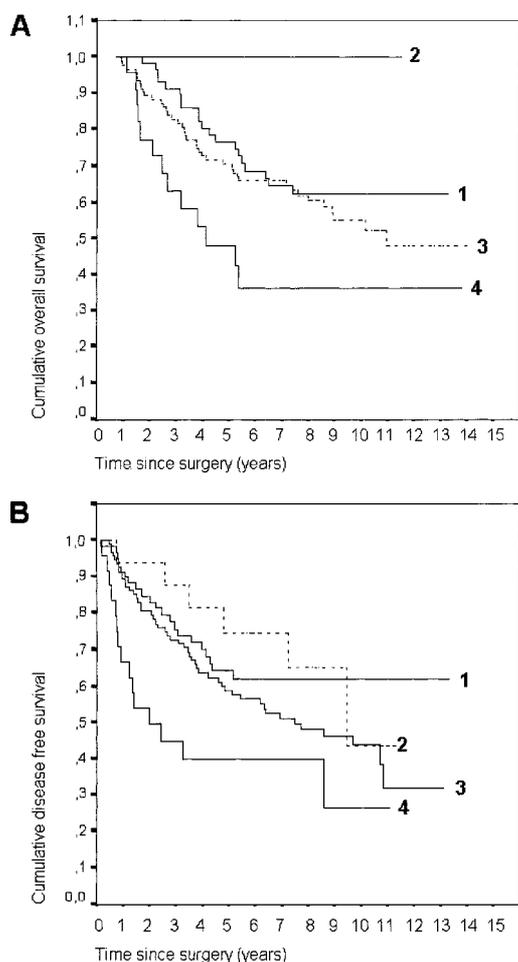


Fig. 3 A, cumulative overall survival of 206 breast cancer patients with absent or weak HIF-1 α expression/absent p53 expression ($n = 65$; Line 1), absent or weak HIF-1 α expression/positive p53 expression ($n = 19$; Line 2), moderate or strong HIF-1 α expression/absent p53 expression ($n = 97$; Line 3), and moderate or strong HIF-1 α expression/positive p53 expression ($n = 25$; $P = 0.0005$, log-rank test; Line 4). B, cumulative DFS of 206 breast cancer patients with absent or weak HIF-1 α expression/absent p53 expression ($n = 65$; Line 1), absent or weak HIF-1 α expression/positive p53 expression ($n = 19$; Line 2), moderate or strong HIF-1 α expression/absent p53 expression ($n = 97$; Line 3), and moderate or strong HIF-1 α expression/positive p53 expression ($n = 25$; $P = 0.0078$, log-rank test; Line 4).

weak HIF-1 α expression. HIF-1 α also remained a significant factor in multivariate analysis. Therefore, immunostaining for HIF-1 α might serve as a biomarker for a more aggressive behavior of human breast cancers, significantly contributing to a more refined assessment of clinical outcome.

In a previous study, Bos *et al.* (17) already suggested that HIF-1 α may play a role in the progression of early breast carcinogenesis because of the fact that they observed a correlation of HIF-1 α expression with the histological grade of DCIS. Interestingly enough, in our study there was no correlation of tumor grade with HIF-1 α expression. Most likely, this correlation vanishes with progression to invasive disease. A possible explanation for this phenomenon might be the contention that in

invasive disease, the role of HIF-1 α may be different from that in noninvasive disease.

It is clear that several tumor suppressor genes and oncogenes show an altered function in invasive disease. Therefore, it is important to reveal combinations of phenotypic changes involving one or more proteins showing a predictable biological behavior. In this study, two proteins considered to be of central importance in the biology of breast cancer did not show any interrelationship with HIF-1 α overexpression; there was no significant correlation of HIF-1 α and HER-2 expression supporting the findings of Bos *et al.* (17). Furthermore, there was no interrelationship of estrogen receptor density with HIF-1 α expression. This latter finding is in contrast to results of Bos *et al.* (17), who found a positive correlation of HIF-1 α and estrogen receptor expression. Therefore, the relationship of estrogen receptor to HIF-1 α deserves additional investigations.

HIF-1 α is considered to support tumor growth through induction of angiogenesis via, *e.g.*, transactivation of the *VEGF* gene. On the other hand, HIF-1 α was reported to associate with p53 protein, thus increasing the stability of p53 (15). In this situation, cells have a higher susceptibility to succumb because of hypoxia through p53-induced apoptosis, thus inhibiting tumor growth. The fact that HIF-1 α may support hypoxia-mediated apoptosis via stabilization of p53 is supported by two findings: (a) loss of wild-type p53 is associated with a marked reduction in hypoxia-mediated apoptosis (28); and (b) HIF-1 α ^{-/-} embryonal stem cells show no induction of p53 protein or apoptosis in response to O₂ and glucose deprivation (14). Therefore, the combination of p53 protein dysfunction, *e.g.*, through somatic mutation, and HIF-1 α overexpression seems to promote tumor progression, *e.g.*, by increased neoangiogenesis in combination with the loss of the proapoptotic function of HIF-1 α (29). This notion is supported by our findings in ovarian cancers, where the combination of HIF-1 α and p53 overexpression was an indicator of a dismal prognosis. In this subgroup, the apoptotic index of tumor cells was low, whereas neoangiogenesis, as assessed by microvessel density, was increased (11). In this study, the combination of p53 and HIF-1 α overexpression, which was found in ~10% of advanced-stage breast cancers, was also associated with a very unfavorable prognosis in univariate analysis. Nevertheless, no significant influence of this combination on survival was found in multivariate analysis.

In p53-positive cases, expression of p53 was not a prerequisite for HIF-1 α expression, indicating that regulation of HIF-1 α expression cannot be attributed to p53 alone. This thesis is further supported by the fact that a subset of cells showed HIF-1 α but not p53 expression. Nevertheless, at least some of these cells might represent tumor-associated macrophages, which are known to express HIF-1 α (8) but not p53. Another interrelationship of p53 and HIF-1 α was reported in earlier studies revealing that p53 may be implicated in the degradation of HIF-1 α (29). Thus loss of p53 function may result in augmented HIF-1 α levels (9). In this study, however, there was no correlation between positivity for p53 and strong HIF-1 α expression, a finding that is supported by the results of Bos *et al.* (17) and our previous findings in ovarian cancer (11) but which is in contrast to results of another study (7).

In conclusion, HIF-1 α is expressed in the vast majority of human breast cancers and is of prognostic significance. Re-

cently, various substances have been demonstrated to interact with HIF-1 α in tumor cells, thus providing a basis for the development of future therapeutic agents targeting HIF-1 α (30–32). Our results suggest that patients with advanced-stage breast cancer might profit from these therapies specifically targeting and inhibiting HIF-1 α . Also, gene therapy driven by hypoxia responsive elements may have good access to breast cancers expressing HIF-1 α and may be switched on by HIF-1 (33).

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Note Added in Proof

A very recent report (Beasley *et al.*, *Cancer Res.*, 62: 2493–2497, 2002) demonstrated that overexpression of HIF-1 α was associated with better survival in patients with head and neck cancer. This finding indicates that, although HIF regulates many genes that enhance tumor growth, the overall balance of activation effects and so the impact on clinical outcome may depend on the type of cancer and treatment modality.

APPENDIX

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