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Advances in Brief

Overexpression of Hypoxia-inducible Factor 1α Is a Marker for an Unfavorable Prognosis in Early-Stage Invasive Cervical Cancer[1]

Peter Birner,[2] Monika Schindl, Andreas Obermair, Christina Plank, Gerhard Breitenecker, and Georg Oberhuber

Institute of Clinical Pathology [P. B., C. P., G. B., G. O.] and Department of Gynecology and Obstetrics [M. S., A. O.], University of Vienna, A-1090 Vienna, Austria

Abstract

Hypoxia-inducible factor 1α (HIF-1α) is a transcriptional factor that regulates genes involved in response to hypoxia and promotes neoangiogenesis, which are considered essential for tumor growth and progression. Using immunohistochemistry, we investigated the influence of HIF-1α expression on prognosis in 91 patients with cervical cancer stage pT1b. In univariate and multivariate analysis, patients with strong expression of HIF-1α had a significantly shorter overall survival time ($P = 0.0307$, log-rank test) and disease-free survival time ($P < 0.0001$, log-rank test) compared with those with moderate to absent HIF-1α expression. HIF-1α expression is a strong independent prognostic marker in early stage cervical cancer.

Introduction

It is well known that in the absence of neovascularization, growth of malignant epithelial tumors is limited to several mm^3, because of the restricted capacities of oxygen and/or glucose diffusion from blood vessels (1). After having reached this size, the tumor volume is only subject to minor changes as the rate of cell death largely equals that of cell division. Several factors assist carcinomas in surmounting its limits in tumor growths: (*a*) the development of vessels. As a matter of fact, angiogenesis is considered essential for tumor growth and the development of metastases (2) and the progression from precursor lesions to invasive cancer (3); and (*b*) the cellular adaptation to hypoxia (4, 5). This is particularly important because cancer cell proliferation may outpace the rate of angiogenesis (1), resulting in tissue hypoxia, thus forcing the tumor to adapt to these environmental conditions.

The mechanisms leading to adaptation of tumor cells to these unfavorable environmental factors are still poorly understood (5). One key factor in supporting adaptation may be HIF-1,[3] which is known to play an essential role in cellular O_2 homeostasis (6). HIF-1 is a heterodimeric bHLH-PAS complex [PAS is an acronym that refers to the first proteins in which this motif was identified, *i.e.*, PER (the protein product of the *Drosophila period* gene), ARNT (the aryl hydrocarbon receptor nuclear translocator), and SIM (the protein product of the *Drosophila single-minded* gene)] composed of the two subunits HIF-1α and HIF-1β (7). The bHLH domain mediates dimerization and DNA binding in a large number of transcription factors. PAS is an additional dimerization motif. Whereas HIF-1β is a common subunit of multiple bHLH proteins, HIF-1α is the unique, O_2-regulated subunit that determines HIF-1 activity (6).

Cervical cancer is one of the most common cancers in women worldwide (8). Because of nationwide screening programs in developed countries, most patients are first seen with stage 1 disease. Stage 1 cervical cancer has a favorable outcome in most patients; nevertheless, approximately 20–35% of patients are expected to die from their disease (9). Until now, it is unknown which factors influence the fate of these patients. Overexpression of HIF-1α protein has been demonstrated in a variety of human cancers by immunohistochemistry (5); data on its impact on prognosis, in particular in cervical cancer, do not exist thus far. The aim of our study was to investigate the impact of immunohistochemically detected HIF-1α expression on prognosis in human early-stage cervical cancer.

Materials and Methods

Patients and Tissues. Formalin-fixed, paraffin-embedded surgical specimens of 91 patients with invasive cervical cancer, UICC stage pT1b, were examined. Diagnosis was established preoperatively by punch biopsy or cone excision, and patients were treated with radical hysterectomy and pelvic lymph node dissection. In cases with pelvic lymph node metastases or tumor invasion of the outer third of the uterine cervix, adjuvant radiation therapy was applied postoperatively. Radiation therapy consisted of brachytherapy at a total dose of 42 Gy applied intracavitarily. In patients with positive lymph nodes, external beam radiation at a total dose of 50 Gy was applied. The mean observation time was 81.6 $\pm$ 42.7 months. During this observation period, 29 patients (31.9%) developed recurrent disease and deceased.

Tumors were considered bulky when infiltrating the outer third of the cervix or having a diameter of 40 mm or more. Vascular space involvement was determined in slides routinely stained with H&E and was considered positive if at least one tumor cell cluster was clearly visible in a vascular space (10). Tissue of five cervices histologically considered normal were used to compare HIF-1α expression in normal *versus* malignant epithelia. In addition, 10 samples of CIN III were also immunostained for HIF-1α (mean patient age, 37.5 $\pm$ 10.5 years).

Immunohistochemistry. The expression of HIF-1α was determined immunohistochemically in paraffin-embedded specimens fixed in 4% buffered formalin. Histological slides, 4 μm in thickness, were deparaffined in xylol. Slides were heated in 0.01 M citrate buffer for 16 min in a microwave oven, and endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 min. For immunohistochemical detection of HIF-1α, specimens were incubated overnight at 4°C with a monoclonal anti-HIF-1α antibody (Clone MAb H1α67, #NB 100–105; Novus Biologicals, Littleton, CO; Ref. 5) in a dilution of 1:60. According to the manufacturer's specifications, this antibody recognizes bands at M_r 120,000 in Western blot, representing HIF-1α in activated cells, and has also been used to immunoprecipitate human HIF-1α. Visualization of bound antibodies was performed by using a Super Sensitive kit (#AP900-M; BioGenex, San Ramon, CA), which is based on streptavidin-biotin-horseradish peroxidase complex formation, according to the manufacturer's instructions. As chromogen, 3-amino-9-ethylcarbazole (BioGenex) was used. As positive control, a specimen of colon adenocarcinoma with strong expression of HIF-1α was used (5).

Expression of HIF-1α was determined by two independent observers (P. B. and G. O.) by assessing semiquantitatively the percentage of stained tumor cells and the staining intensity. The percentage of positive cells was rated as

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[2] To whom requests for reprints should be addressed, at the Institute of Clinical Pathology, University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. Phone: 43-1-40400-3650; Fax: 43-1-4053402; E-mail: peter.birner@akh-wien.ac.at.

[3] The abbreviations used are: HIF-1, hypoxia-inducible factor 1; bHLH, basic helix-loop-helix; UICC, International Union Against Cancer; CIN, cervical intraepithelial neoplasia; OS, overall survival; DFS, disease-free survival; VEGF, vascular endothelial growth factor; HPV, human papillomavirus.

follows: 2 points, 11–50% positive tumor cells; 3 points, 51–80% positive cells; and 4 points, >81% positive cells. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for expression and percentage of positive cells were added, and specimens were attributed to four groups according to their overall score: negative, ≤10% of cells stained positive, regardless of intensity; weak expression, 3 points; moderate expression, 4–5 points; and strong expression, 6–7 points.

Expression of p53 was investigated using a standard protocol with the monoclonal antibody DO-7 (Dako, Glostrup, Denmark; Ref. 11). Detection was performed using a Super Sensitive kit (#AP900-M; BioGenex) and diaminobenzidine. As positive control, a specimen of colorectal cancer with a known mutation of p53 was used. A specimen was considered as "positive" for p53 expression if a vast majority of tumor cells showed distinct nuclear staining, suggesting accumulation of nonfunctional p53, otherwise as "negative" with regard to p53 expression. The negative control slides for both antibodies were prepared from the same tissue block. Instead of the primary antibody, a nonimmune serum was applied.

Statistical Methods. Differences in HIF-1α expression between cervical cancer and CIN III were investigated using the Mann-Whitney- test, as well as differences in HIF-1α expression between tumors with and without expression of p53. Correlation of HIF-1α expression with various clinical and histopathological parameters was investigated using Kruskal-Wallis test. OS was defined as the period from primary surgery until the death of the patient. Death from a cause other than cervical cancer or survival until the end of the observation period were considered censoring events. DFS was defined from the end of primary therapy until first evidence of progression of disease. Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier (12). The Cox proportional- hazards model was used for multivariate analysis. HIF-1α expression, lymphatic node status, vascular invasion, histological grading, and tumor size (bulky *versus* nonbulky) were entered into Cox regression. For all tests, a two-tailed $P \leq 0.05$ was considered significant.

Results

HIF-1α expression was recognized through a nuclear staining of positive cells. There was no expression of this protein in normal cervical samples. In contrast, immunohistochemistry revealed decoration by the HIF-1α antibody in 81.3% of the tumor samples. In 16 (17.6%) cases, there was a weak (Fig. 1), in 38 (41.8%) a moderate, and in 20 samples (22%) a strong expression of HIF-1α (Fig. 2). In only 17 samples (18.7%), no HIF-1α was detected in tumor cells. In 77 cases, nondysplastic squamous cell epithelium directly adjacent to invasive cancer was present, and in 15 cases, it showed weak expression of HIF-1α in the basal and intermediate cells. In these cases, the cancer cells also showed moderate to strong expression of HIF-1α.

Two specimens with CIN III showed no expression of HIF-1α, 2 weak, 4 moderate, and 2 strong expression of HIF-1α. No significant difference between HIF-1α expression between cervical cancer and CIN III was observed ($P = 0.83$, Mann-Whitney test).

In the Kruskal-Wallis test, no significant correlation between HIF-1α expression and lymphatic node involvement ($P = 0.325$), patients' age ($P = 0.442$), histological grading ($P = 0.105$), and tumor size ($P = 0.499$) was found. Ten specimens of invasive cancer (11%) were considered positive with regard to p53 expression, and 2 specimens (20%) of CIN III. No correlation of p53 and HIF-1α expression was observed ($P = 0.453$, Mann-Whitney test).

When survival of patients with strong expression of HIF-1α was compared with survival of patients with moderate to absent expression of HIF-1α, Kaplan-Meier analysis (log-rank test) revealed a significant influence of HIF-1α expression on OS ($P = 0.0307$; Fig. 3*A*) and DFS ($P < 0.0001$; Fig. 3*B*). Expression of HIF-1α remained an independent prognostic factor for OS ($P = 0.0129$) and DFS ($P = 0.0002$) in multivariate analysis (Table 1).

The 5-year OS rate was 83% in patients with moderate to absent expression of HIF-1α (median OS time, 103 months), whereas in patients with strong HIF-1α expression, it was 75% (median OS time, 85 months). The 5-year DFS rate was 80% in patients with moderate to absent expression of HIF-1α (median DFS time, 170 months), whereas in patients with strong HIF-1α expression, it was only 34% (median DFS time, 28 months).

Discussion

Here we show that expression of HIF-1α as revealed by immunohistochemistry can be observed in various intensities in 81.3% of early-stage invasive cervical cancers and in 80% of CIN III but not in "normal" cervical epithelia. We demonstrate for the first time that HIF-1α expression is a strong prognostic marker mainly for DFS in UICC stage pT1b cervical cancer.

How can this influence on prognosis be explained in cervical cancer? HIF-1α probably has a dual function in early cancerogenesis. On the one hand, it may stimulate angiogenesis via transactivation of the *VEGF* gene, thus supporting tumor growth. On the other hand, HIF-1α may associate with p53 protein, thus increasing the stability of p53 (13). In this situation, cells have a higher susceptibility to succumb because of hypoxia through p53-induced apoptosis. This is supported by the fact that the loss of wild-type p53 is associated with a marked reduction in hypoxia-mediated apoptosis (14). The interrelationship between HIF-1α and p53 is also supported by the finding

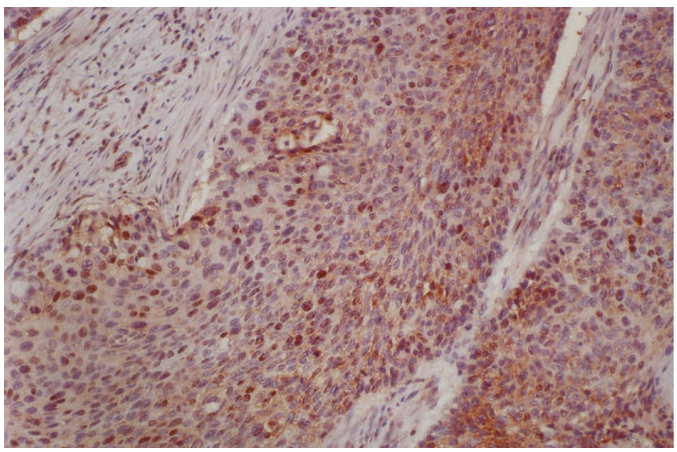

Fig. 1. A specimen of invasive cervical cancer with weak expression of HIF-1α. Nuclear staining of weak intensity can be seen only in a subset of cancer cells. Immunoperoxidase, ×200.

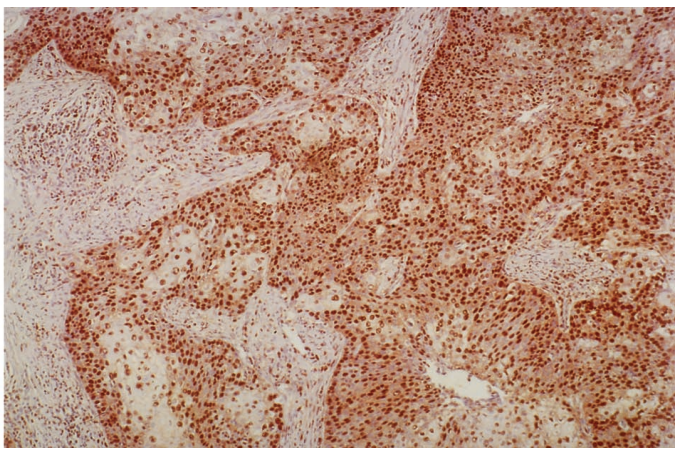

Fig. 2. A specimen of invasive cervical cancer with strong expression of HIF-1α. Note the distinct nuclear staining in a vast majority of cancer cells. Immunoperoxidase, ×100.

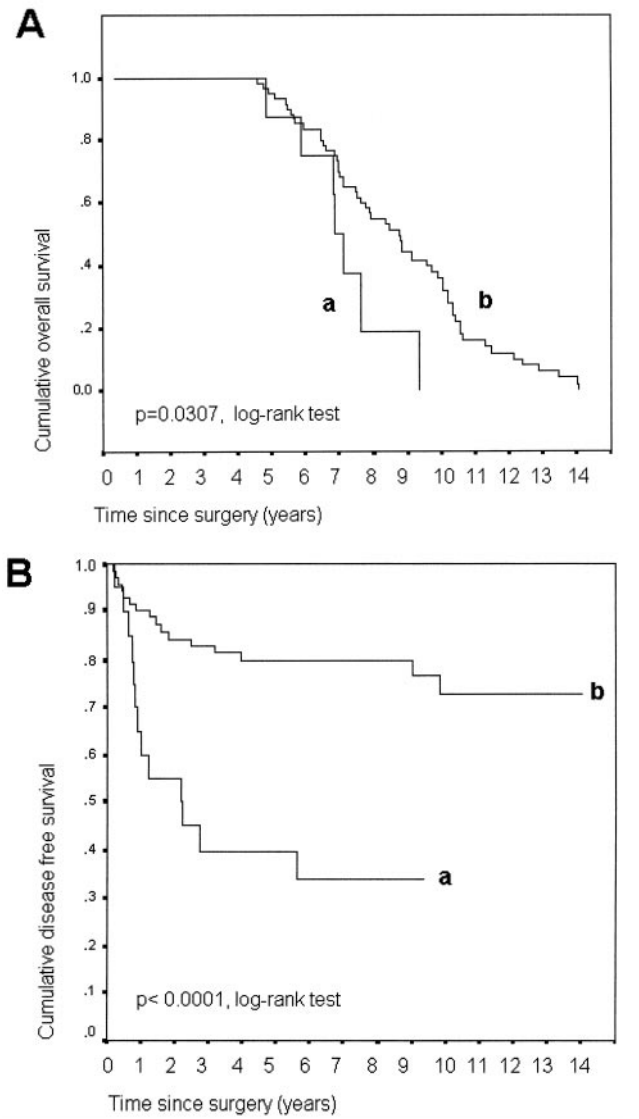

Fig. 3. *A*, cumulative OS in 91 patients with cervical cancer stage pT1b with strong expression of HIF-1α (*a*) and moderate to absent expression of HIF-1α (*b*). *B*, cumulative DFS in 91 patients with cervical cancer stage pT1b with strong expression of HIF-1α (*a*) and moderate to absent expression of HIF-1α (*b*).

Table 1 *OS and DFS in 91 patients with cervical cancer stage pT1b (Cox regression)*

	Significance (*P*)	95% Confidence interval	Relative risk
OS			
HIF-1α expression	0.0129	1.25–6.66	2.89
Lymph node involvement	0.0005	1.92–10.03	4.38
Tumor size	0.5008		
Grading	0.0218	1.1–3.32	1.91
Vascular invasion	0.0049	1.35–5.32	2.68
DFS			
HIF-1α expression	0.0002	2.16–11.7	5.04
Lymph node involvement	0.0001	2.47–14.71	6.03
Tumor size	0.1099		
Grading	0.4925		
Vascular invasion	0.0755		

that HIF-1α−/− ES cells show no induction of p53 protein or apoptosis in response to O_2 and glucose deprivation (15). Therefore, HIF-1α may support hypoxia-mediated apoptosis via stabilization of p53. It therefore appears that the combination of p53 protein dysfunction, *e.g.*, through somatic mutation, and HIF-1α overexpression seem to be necessary to allow HIF-1α to sufficiently stimulate tumor progression in early cancerogenesis through mediating angiogenesis and inducing adaptive intracellular responses to hypoxia without supporting proapoptotic mechanisms.

In the vast majority of patients, cervical cancer is caused by infection with HPV types with high oncogenic risk (*e.g.*, HPV 16, 18, and 33; Ref. 16). HPV may be an important coplayer for the development of tumorigenic properties of HIF-1α. In cervical cancer, p53 is most commonly inactivated by the viral oncoprotein E6 (17) and not by mutations (18). Furthermore, the E6 oncoprotein has been demonstrated to stimulate HIF-1α expression as a consequence of ubiquitin-dependent conjugation and degradation of p53 (19). Therefore, in cervical cancer, the tumor-suppressive functions of HIF-1α may be lost already in initial stages in the majority of cases through the influence of HPV infection and p53 protein inactivation. The function remaining is its angiogenic property via activation of the *VEGF* gene (19). The extent of neoangiogenesis, as assessed by determination of microvessel density, is influenced by VEGF (20) and is considered to support progression of cervical cancer (3). Consistent with this notion are results of earlier studies on the collective presented here showing that a high microvessel density had a negative impact on survival (9).

Presumably because of HPV infection, the impact of HIF-1α expression on prognosis may be more clearly seen in cervical cancer as compared with other cancers with functioning p53 in a high percentage of cases. On the basis of our findings, we speculate that increased expression of HIF-1α is an important event in the progression of cervical cancer at least in a subgroup of patients. It may either increase O_2 availability or metabolic adaptation to O_2 deprivation. This notion is supported by the fact that HIF-1α influences a number of genes that partly play a role in tumor progression including *erythropoietin, transferrin, endothelin-1, inducible nitric oxide synthetase, heme oxygenase 1, VEGF, insulin-like growth factor-2, insulin-like growth factor-binding proteins -2 and -3*, and 13 different glucose transporters and glycolytic enzymes (6).

In conclusion, we have shown here for the first time that overexpression of HIF-1α is a marker for tumor progression in cervical cancer. Further studies will show whether HIF-1α has a similar impact on prognosis in other forms of cancer. Furthermore, they should reveal whether inactivation of p53, as it is known to exist in cervical cancer through the viral protein E6, is essential for the potential tumorigenic effects of HIF-1α.

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