Overexpression of Hypoxia-inducible Factor 1α Indicates Diminished Response to Radiotherapy and Unfavorable Prognosis in Patients Receiving Radical Radiotherapy for Cervical Cancer

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ABSTRACT

Purpose: The purpose is to investigate the impact of hypoxia-inducible factor (HIF)-1α expression on response to radiotherapy and prognosis of patients with primary irradiated cervical cancer. Because human papillomavirus (HPV) oncoprotein E6 might interact with HIF-1α in various pathways, we also investigated the relation of HIF-1α and HPV status.

Experimental Design: Expression of HIF-1α was investigated by immunohistochemistry in 67 specimens of patients who had received radical radiotherapy for cervical cancer stages IB–IIIB. HPV analysis was performed using type-specific PCR, cloning, and sequencing. Survival analysis was performed using univariate and multivariate analysis.

Results: Immunohistochemistry revealed expression of HIF-1α in 72.1% of the tumor samples. In 16 (23.9%) cases, there was a weak expression, in 25 (37.3%) a moderate expression, and in 7 cases (10.4%) a strong expression of HIF-1α. Nineteen samples (28.4%) were considered negative for HIF-1α expression. Strong/moderate expression of HIF-1α was associated with only partial response to radiotherapy (P = 0.037, χ² test). Strong/moderate expression of HIF-1α was also an independent prognostic factor for shorter progression-free survival (P = 0.036, Cox regression) and cervical cancer-specific survival (P = 0.04, Cox regression). No association between HIF-1α expression and infection with different HPV types could be found.

Conclusions: Overexpression of HIF-1α has predictive and prognostic significance in cervical cancer patients receiving curative radiation therapy. Possibly, expression of HIF-1α could serve as intrinsic marker of hypoxia in cervical cancer.

INTRODUCTION

Angiogenesis is considered as essential for growth and progression of solid malignant tumors (1). If this formation of new blood vessels is not sufficient to provide enough O₂ to proliferating tumor cells, tissue hypoxia results. However, a dense network of newly formed capillaries within tumors does not necessarily imply that these capillaries are fully functional, and thus hypoxia is not present (2). Therefore, tissue hypoxia is a common feature of most solid tumors, often with heterogeneous O₂ levels within different regions of the individual tumors.

Tissue hypoxia within malignant tumors is considered an important factor for response to treatment. Hypoxic tumor cells are resistant to radiation therapy (3), and in cervical cancer treated with radiotherapy, low oxygen tension assessed by polarographic oxygen needle electrodes are associated with increased rate of metastasis and poor survival (4–7).

The cellular adaptation to hypoxic stress is highly complex and depends on the regulation of genes supporting anaerobic metabolism and new blood vessel recruitment. The transcription factor HIF-1α is a key factor in this adaptation (8, 9).

We have recently demonstrated that HIF-1α is closely associated with dismal prognosis in early-stage cervical cancer treated by primary surgery (10). Because HIF-1α has been recently shown to be associated with response to radiotherapy in oropharyngeal cancer (11), head and neck cancer (12), in early esophageal cancer (13), and in nasopharyngeal carcinomas (14), the aim of this study was to investigate the impact of HIF-1α expression on response to radiotherapy and prognosis of patients with primary irradiated cervical cancer. This is of particular interest because extensive tissue hypoxia is considered as a main cause for treatment failure in radio-oncology (15).

A strong causal relationship between infection with HPV and cervical cancer has been established (16), and the viral
oncoproteins E6 of various HPV types differ in oncogenic potential (14). Because E6 might interact with HIF-1α in various pathways (17, 18), we also investigated the relation of HIF-1α expression and HPV status in our collective of patients.

MATERIALS AND METHODS

Patients. Formalin-fixed, paraffin-embedded biopsy samples of 67 patients with primary cervical cancer FIGO stage IB–IIIB were included in the study. All patients underwent primary radiotherapy at the Department of Radiotherapy and Radiobiology, University Hospital of Vienna, between November 1993 and October 2001.

Bulky disease was defined as (a) visible cervical tumor with the largest diameter >4 cm or (b) a cervix expanded to >4 cm as a result of tumor invasion. Computerized topography of the pelvis and abdomen was performed to determine the nodal status. Patients with enlarged para-aortic and/or common iliac nodal involvement were considered lymph node positive.

All patients underwent primary radiotherapy with the intention of achieving cure. The treatment consisted of external beam radiotherapy with a four-field box technique to the pelvis and to the para-aortic region (depending on the stage), consisting of a total dose of 40–50 Gy (median, 48.6 Gy) applied in daily fractions of 1.6–2 Gy. External beam irradiation was carried out using a 25-MV linear accelerator, with central shielding in anterior-posterior portals, depending on the stage of disease and tumor volume. Three to six fractions of intracavitary high dose-rate brachytherapy were applied in weekly fractions of 7 Gy each to point “A,” depending on the tumor stage and tumor volume (19).

The follow-up investigations were commenced 4 weeks after the last treatment and were performed thereafter at 3-month intervals. Response to treatment was evaluated by clinical examination and by appropriate imaging studies (in all cases computerized topography, whenever appropriate, magnetic resonance imaging and ultrasound) at 3 months. Persistent disease was defined as disease within the pelvis 3 months after completion of radiotherapy. Recurrent disease was defined as local (within pelvis) or distant (outside the pelvis).

Immunohistochemistry. For immunohistochemical detection of HIF-1α, a 4-μm tissue section was deparaffinized in xylene followed by microwave treatment in for 30 min in 0.01 M citrate buffer (pH 6.0) at 600 W. 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. Specimens were incubated overnight at +4°C with a monoclonal anti-HIF-1α antibody (no. H72320; BD Transduction Laboratories, Franklin Lakes, NJ; Refs. 20, 21) at a dilution of 1:25. Detection of immunostaining was performed using the ChemMate kit (Dako, Glostrup, Denmark) and 3,3′-diaminobenzidine as chromogene. For positive control, HIF-1α immunostaining was also performed on two samples of ovarian cancer with known strong expression, which have also been used in a previous study (22). For negative control, the primary antibody was replaced by nonimmune isotypic antibodies.

Nuclear expression of HIF-1α was determined by assessing semiquantitatively the percentage of decorated tumor cells and the staining intensity (11, 22). The percentage of positive cells was rated as follows (11, 22): cases with ≤10% positive cells were rated as negative (no points attributed), 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, ≤10% of cells stained positive, regardless of intensity; weak expression, 3 points; moderate expression, 4–5 points; and strong expression, 6–7 points. Two independent investigators blinded to clinical data performed the analysis. Specimens scored differently by the two investigators were reinvestigated together using a multisampled microscope. In addition, the presence of necrotic areas within tumor formations and HIF-1α expression in tumor cells directly adjacent to these areas was evaluated.

HPV Analysis. DNA was extracted from 10-μm thick paraffin-embedded tissue sections of 64 cases for HPV-analysis as described previously (23, 24). In 3 cases, not enough material was left for analysis. All samples were tested for β-globin (25), which served as internal control for the integrity of the extracted template, followed by consensus HPV-PCR (GP5+/GP6+; Ref. 26) from the L1 region.

Typing of HPV DNA was performed by type-specific PCR: HPV-16 (E7/E1: 698–917); HPV-18 (E6/E7: 533–705; Ref. 27); HPV-31 (E5: 3835–3989; Ref. 27); HPV-33 (E7: 610–840; Ref. 27); HPV-33 (E6: 265–396); and HPV-45 (E6/E7: 548–694).

After PCR amplification, the PCR products were separated on a 2% Tris-acetate-EDTA-agarose gel, followed by visualization of the DNA fragments under UV light.

To detect infection with other HPV types, consensus PCR products were cut out and purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The purified consensus HPV DNA was cloned into pTargetTTM Mammalian Expression Vector System (Promega, Madison, WI), followed by thermocycle sequencing (Amersham Life Science, Piscataway, Ohio).

As additional positive control, we investigated HPV status in selected cases with signal-amplified in situ hybridization with probes against HPV-16, HPV-18, HPV-31, and HPV-33 as described previously (28).

Statistical Methods. For statistical analysis, two groups of patients were formed with regard to HIF-1α expression: i.e., absent or low expression and strong or moderate expression.

Association between HIF-1α expression and clinicopathological factors were analyzed by using Mann-Whitney test and the χ² test.

PFS was defined as the period from end of therapy to the date of the first documented evidence of recurrent disease. CCSS was calculated from the date of diagnosis to death; patients who survived until the end of the observation period were censored at their last follow-up visit. Patients who died because of other causes than cervical cancer were censored at their date of death. Survival curves were calculated using Kaplan-Meier estimates, and differences between groups were tested by log-rank test. Multivariate survival analysis was per-
formed according to the Cox proportional hazards model. HIF-1α expression (absent/low versus moderate/strong), tumor size (bulky versus nonbulky), patients’ age, nodal status, FIGO stage, and histological grading were included in the regression model.

For all statistical tests, \( P \leq 0.05 \) was considered significant.

**RESULTS**

Clinical and histopathological patient characteristics are given in Table 1. Fifty-five patients had a complete clinical response to radiotherapy, whereas 12 patients (17.9%) had incomplete response \( (n = 7) \) or lymphogene disease progression \( (n = 5) \) after radiation therapy. Median follow-up time was 27 months (range, 5–84 months). During this observation period, 23 patients experienced both local and/or distant relapse, and 35 patients (all patients with incomplete response to radiotherapy and all patients with recurrent disease) died because of cervical cancer.

**HIF-1α Expression.** Immunohistochemistry revealed decoration by the HIF-1α antibody in 71.6% of the tumor samples. In 16 (23.9%) cases, there was a weak expression, in 25 (37.3%) a moderate expression, and in 7 cases (10.4%) a strong expression of HIF-1α (Fig. 1). Nineteen samples (28.4%) were considered as negative for HIF-1α expression. Twenty-nine tumors (43.3%) were found to have necrotic areas, and 27 of these tumors (93.1%) showed perinecrotic HIF-1α expression. Tumors with necrotic areas had significantly more often strong/moderate HIF-1α expression compared with the other cases (median expression: moderate versus weak, \( P < 0.001 \), \( \chi^2 \) test; Table 1). No significant association between

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**Table 1** Patient characteristics and HIF-1α expression

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>HIF-1α expression</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Strong/moderate</td>
</tr>
<tr>
<td></td>
<td>( n )</td>
<td>( n )</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>6 ( 8.9 )</td>
<td>3 ( 50 )</td>
</tr>
<tr>
<td>IIa</td>
<td>34 ( 50.8 )</td>
<td>15 ( 44.1 )</td>
</tr>
<tr>
<td>IIIb</td>
<td>27 ( 40.3 )</td>
<td>14 ( 51.9 )</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4 cm</td>
<td>19 ( 28.4 )</td>
<td>8 ( 42.1 )</td>
</tr>
<tr>
<td>&gt;4 cm</td>
<td>48 ( 71.6 )</td>
<td>24 ( 50 )</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>59 ( 88.1 )</td>
<td>27 ( 45.8 )</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>8 ( 11.9 )</td>
<td>5 ( 62.5 )</td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With necrosis</td>
<td>29 ( 43.3 )</td>
<td>22 ( 75.9 )</td>
</tr>
<tr>
<td>Without necrosis</td>
<td>38 ( 56.7 )</td>
<td>10 ( 26.3 )</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
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<tr>
<td>Negative</td>
<td>46 ( 68.7 )</td>
<td>20 ( 43.5 )</td>
</tr>
<tr>
<td>Positive</td>
<td>21 ( 31.3 )</td>
<td>12 ( 57.1 )</td>
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<tr>
<td>Grading</td>
<td></td>
<td></td>
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<tr>
<td>Grade 1</td>
<td>7 ( 12.1 )</td>
<td>2 ( 28.6 )</td>
</tr>
<tr>
<td>Grade 2</td>
<td>34 ( 58.6 )</td>
<td>17 ( 50 )</td>
</tr>
<tr>
<td>Grade 3</td>
<td>17 ( 29.3 )</td>
<td>8 ( 47.1 )</td>
</tr>
<tr>
<td>HPV status</td>
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<td></td>
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<tr>
<td>Negative</td>
<td>6 ( 8.9 )</td>
<td>2 ( 33.3 )</td>
</tr>
<tr>
<td>Single HPV-16</td>
<td>20 ( 29.9 )</td>
<td>9 ( 45 )</td>
</tr>
<tr>
<td>Other single HPV types</td>
<td>10 ( 14.9 )</td>
<td>4 ( 40 )</td>
</tr>
<tr>
<td>Multiple HPV-16 + HPV-33</td>
<td>16 ( 23.9 )</td>
<td>9 ( 60 )</td>
</tr>
<tr>
<td>Other multiple HPV types</td>
<td>12 ( 17.9 )</td>
<td>3 ( 50 )</td>
</tr>
<tr>
<td>HPV positive/subtype unknown</td>
<td>3 ( 4.5 )</td>
<td>3 ( 100 )</td>
</tr>
</tbody>
</table>

\* IIa, \( n = 3 \); IIB, \( n = 31 \).
\* IIIa, \( n = 9 \); IIIb, \( n = 18 \).
HIF-1α expression and FIGO stage (P = 0.664), tumor size (P = 0.563), histology (P = 0.377), lymphatic node involvement (P = 0.303), and histological grading (P = 0.619) was found (Table 1).

Association of HPV Infection and HIF-1α Expression.
HPV DNA was detected in 58 of 64 (91.6%) of the patients. Thirty of these patients (51.7%) had tumors with one HPV DNA genotype, and 28 patients (48.3%) had tumors with two or more HPV DNA genotypes.

HPV-16 was the most commonly found genotype and was detected in 42 cases of HPV-positive tumors. HPV-16 was found in 20 patients as the only HPV type (47.6%) and in 22 patients’ cases as part of multiple HPV infection (52.4%). HPV-33 was the second most common genotype (n = 24) and was found in 2 patients as a single HPV type (8.3%) and in 22 additional patients as part of multiple HPV types (91.7%). The most commonly found combination of multiple HPV types involved HPV-16 plus HPV-33 (n = 15). Other high-risk types (single and/or multiple HPV types) included HPV-18 (7 patients), HPV-31 (8 patients), HPV-45 (4 patients), and HPV-58 (1 patients) and the low-risk types HPV-73 (2 patients) and HPV-69 (1 patient).

For analyzing the influence of various HPV-types on HIF-1α expression, HPV types were grouped as follows: no HPV infection, n = 6 (10.3%); single HPV-16, n = 20 (34.5%); other single HPV infection, n = 10 (17.2%); HPV-16 + HPV-33, n = 16 (26.6%); and other multiple HPV infection, n = 12 (20.7%). No association of HIF-1α expression and infection with various HPV subtypes was observed (P = 0.29, Mann-Whitney test).

Association of HIF-1α Expression with Response to Therapy. Seventy-five percent (n = 9) of the 12 patients who had an incomplete response to radiotherapy were found to have a strong/moderate HIF-1α expression before commencing radiotherapy, whereas in patients with complete response to radiotherapy (n = 55), strong/moderate HIF-1α expression was found in only 41.8% (n = 23) of cases (P = 0.037; χ² test).

Survival Analysis. When survival of patients with strong/moderate expression of HIF-1α was compared with survival of patients with absent/weak expression of HIF-1α, Kaplan-Meier analysis (log-rank test) revealed a significant influence of HIF-1α expression on PFS (P = 0.011; Fig. 2A) and CCSS (P = 0.006; Fig. 2B). Other factors associated with shortened PFS and CCSS in univariate analysis were tumor size (P < 0.001 and P = 0.004, respectively), positive lymph nodes (P = 0.001 and P = 0.002, respectively), and a more advanced FIGO stage (P = 0.039 and P = 0.034, respectively; Table 2).

The 3-year CCSS rate was 71% in patients with absent/weak expression of HIF-1α (median CCSS time, 62 months), whereas in patients with strong/moderate HIF-1α expression, it was 29% (median CCSS time, 24 months; Table 2).

The 3-year PFS rate was 53% in patients with absent/weak expression of HIF-1α (median PFS time, 60 months), whereas in patients with strong/moderate HIF-1α expression, it was only 34% (median PFS time, 13 months; Table 2).

Expression of HIF-1α was the only independent prognostic factor for PFS (P = 0.049) and CCSS (P = 0.02) in multivariate analysis. To improve the power of the analysis, lymph node status and tumor size were combined as follows: node negative/tumor size ≤ 4 cm, n = 17 (25.4%); node positive/tumor size > 4 cm, n = 2 (3%); node negative/tumor size ≤ 4 cm, n = 29 (43.2%); and node positive/tumor size > 4 cm, n = 19 (28.4%). At multivariate analysis of survival using this combined variable, expression of HIF-1α and the presence of positive node positives combined with tumor size > 4 cm independently predicted outcome, as shown in Table 3.

DISCUSSION
Up to now only a few data exist on the expression of HIF-1α in cervical carcinoma. In an earlier study, we have shown that in patients who underwent radical surgery for early-
stage cervical cancer increased expression of HIF-1α is a strong prognostic marker (10). An association of HIF-1α expression with response to radiotherapy was reported recently for oropharyngeal cancer (11), head and neck cancer (12), early esophageal cancer (13), and nasopharyngeal carcinomas (14).

The present results indicate for the first time a strong association between expression of HIF-1α and response to radiotherapy in cervical cancer patients. In a previous study, Haugland et al. (29) observed a trend to worse prognosis in patients who underwent radical radiotherapy for advanced cervical cancer with strong HIF-1α expression, which did not reach significance. Interestingly, Haugland et al. (29) observed a considerably lower rate of HIF-1α-positive cells (\( \sim 11\% \)) compared with our study using another monoclonal antibody.

Hypoxia is an important factor in many pathological processes, including tumor formation, where it has been associated with resistance to radiotherapy, malignant progression, and metastasis (2–7). Tumors become hypoxic because new blood vessels they develop are aberrant and have poor blood flow (2). Although hypoxia is toxic to both cancer and normal cells, cancer cells undergo genetic and adaptive changes that allow them to survive and even proliferate within a hypoxic environment (30). These processes contribute to the malignant phenotype and to aggressive tumor behavior (31).

Radiotherapy is a major treatment modality for advanced cervical carcinomas and requires free radicals from oxygen to destroy target cells, and cells in hypoxic areas were found to be resistant to radiation-induced cell death (2, 32). In patients with cervical cancer, hypoxia, measured by the use of the Eppendorf probe, has been associated with an increased risk of relapse and death (4–7). Use of the Eppendorf probe as a measure of tumor hypoxia is somewhat cumbersome and expensive. An alternative strategy for the measurements of hypoxia is to use changes in expression of oxygen-regulated proteins.

For this purpose, the transcription factor HIF-1α is an eligible candidate. Although HIF-1α expression might also be induced by oncogenic, not hypoxia-induced stimuli, tissue hypoxia is considered as the main inducer of HIF-1α expression in human tumors (33). This is in good concordance to our findings that HIF-1α expression was associated with the presence of necrotic areas.

HIF-1α is a key transcription factor that was recently demonstrated to be a useful intrinsic marker for hypoxia in cervical cancer xenografts (34). Possibly, expression of HIF-1α might also serve as intrinsic marker of hypoxia in cervical cancer tissue samples.

HIF-1α activates the expression of numerous hypoxia-response genes such as the vascular endothelial growth factor, which promotes angiogenesis, glucose transporter 1, which activates glucose transport, lactate dehydrogenase, which is involved in the glycolytic pathway, and erythropoietin, which induces erythropoiesis. HIF-1α also activates transcription of nitric oxide synthase, which promotes angiogenesis and vasodilatation (35–37).

On the other hand, HIF-1α is also a potent activator of the
p53 tumor suppressor gene to promote p53-dependent apoptosis (38). The potential of HIF-1α to induce apoptosis both in cell culture and in experimental tumors is influenced by the presence of functional p53 (39), and so cells with loss of wild-type p53 are not susceptible to this hypoxia-induced apoptosis. It has been demonstrated that in transplanted tumors expressing wild-type p53, apoptosis correlates strongly with hypoxia, whereas little apoptosis occurs in hypoxic regions of p53-deficient tumors (40).

In cervical cancer, p53 is most commonly inactivated by human papillomavirus oncoprotein E6 (41). E6’s activity with respect to p53 is equivalent to an inactivating mutation of p53 (42), and the inactivation of the tumor suppressor protein p53 by E6 might be a mechanism to evade cell death under hypoxic conditions (43). Oncoproteins E6 of various HPV types differ in their potential of binding to and thus inactivating of p53. The strongest inactivator of p53 is E6 oncprotein of HPV-type 16, followed by E6 of HPV-type 31 and HPV-type 18 (44). Furthermore, the E6 oncoprotein has been demonstrated to stimulate HIF-1α expression as a consequence of ubiquitin-dependent conjugation and degradation of p53 (18), although hypoxia may induce accumulation of p53 by uncoupling its interaction with E6 (45). In this study, we therefore examined the association of HPV infection and HIF-1α expression in advanced cervical cancer to reveal this effect on HIF-1α expression. Our hypothesis was that the relative grade of HIF-1α expression differed in specimens depending on the inactivation potential of the HPV type found in the tissue. However, we have not found such an association. Our results suggest that expression of HIF-1α is not influenced by type-specific HPV oncprotein E6-mediated inactivation of p53. Nevertheless, the viral inactivation of p53 might enhance the oncogenic effects of HIF-1α because its proapoptotic effect is hampered by the nonfunctional p53. Additional studies measuring the quantitative expression of E6 oncprotein in relation to HIF-1α expression would help to clarify the relationship between HIF-1α and HPV.

In summary, we conclude that in cervical cancer treated by radical radiotherapy, overexpression of HIF-1α might serve as a predictive marker for response to this treatment and for diminished prognosis. Future studies will have to show if adaptation of therapy protocols might be of benefit for patients.

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