Expression of Erythropoietin and Erythropoietin Receptor in Cervical Cancer and Relationship to Survival, Hypoxia, and Apoptosis

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Abstract

Purpose: Physiologically, hypoxia induces the expression of erythropoietin (Epo) in adult kidney cells. Epo, in turn, acts on the Epo receptor (EpoR) in RBC precursors to stimulate growth and prevent apoptosis. Because hypoxia plays a major role in the malignant progression of tumors and Epo and its receptors have also been detected in malignant tumors, we investigated the expression of Epo and EpoR and their relationship with hypoxia, proliferation, apoptosis, and clinicopathologic variables in cervical cancer.

Experimental Design: Intratumoral oxygen measurement and needle biopsies of the tumors were done in 48 patients with cervical cancer. The obtained tissue was analyzed by immunohistochemistry with antibodies against Epo, EpoR, and Ki-67 as well as by terminal deoxynucleotidyl transferase–mediated deoxyuracil triphosphate nick-end labeling assays.

Results: Epo and EpoR were expressed in 88% and 92% of samples, respectively. Cervical cancers with higher Epo expression showed a significantly reduced overall survival (3 years, 50.0% versus 80.6%; $P = 0.0084$). Epo and EpoR expression correlated significantly with apoptosis ($r = 0.49$, $P = 0.001$ and $r = 0.36$, $P = 0.021$). Furthermore, EpoR expression correlated significantly with tumor size ($r = 0.32$, $P = 0.032$) and was significantly associated with the presence of lymphovascular space involvement ($P = 0.037$). However, we observed no correlation between Epo or EpoR expression and intratumoral hypoxia, although in well-oxygenated tumors, EpoR localized significantly more often to the invasion front ($P = 0.047$).

Conclusions: This study analyzes Epo/EpoR expression and their relationship with intratumoral pO_2 levels as well as with survival in patients with cervical cancer. The data suggest a critical role of the endogenous Epo/EpoR system in cervical cancer.

Hypoxia plays a major role in the malignant progression of solid tumors. Hypoxic microregions have been detected in a wide variety of solid tumors (1–3). Previous clinical studies done by our group showed that patients with hypoxic cervical cancers had a significantly worse prognosis compared with patients with better oxygenated tumors regardless of treatment modality. Tumor hypoxia was shown to be the most powerful independent prognostic factor in cervical cancer (1). Mechanisms by which sustained tumor hypoxia may increase aggressiveness include differential regulation of gene expression (4) and clonal selection of tumor cells that have lost their apoptotic potential (5, 6). One of the transcription factors most sensitive to a hypoxic micromilieu is the hypoxia-inducible factor 1 (Hif-1; ref. 7). Induction of Hif-1 results in the upregulation of a wide variety of target genes including genes involved in metabolism, angiogenesis, metastasis/invasion, as well as apoptosis (8). A major Hif-1 target gene is erythropoietin (Epo; ref. 9). Epo receptor (EpoR) is also induced by hypoxia but is not regulated by Hif-1 (10, 11).

Epo is a glycoprotein hormone stimulator of erythropoiesis (12) produced in the kidneys and liver, and exerts its effect by stimulating growth, preventing apoptosis, and inducing differentiation of RBC precursors (13). EpoR belongs to the cytokine receptor superfamily (14). Recent studies have detected the expression of Epo and EpoR not only in normal nonhematopoietic tissues and cells, including the central nervous system and vascular endothelial cells (15, 16), but also in a variety of solid tumors (10, 11, 17–22).

Recombinant human Epo is used to treat chemotherapy-induced anemia. Although two large clinical trials documented negative effects of recombinant human Epo on patient outcome in head and neck as well as breast cancer (23, 24), a recent meta-analysis did not find an unfavorable effect on overall survival of the treated cancer patients (25).

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Recently, EpoR was shown *in vitro* to be up-regulated in cancer cell lines under hypoxic conditions (10, 11). However, the relationship between intratumoral pO_2 and the expression of hypoxia-related proteins in human cancer is still not clear. Thus far, the existence of a clear spatial association between hypoxia and the expression of Hif-1 or its targets in clinical cancer samples remains controversial (26–30).

This study investigates the expression of Epo and EpoR and their correlation with intratumoral pO_2 levels in cervical cancer. Furthermore, we studied the association between Epo/EpoR expression and apoptosis, proliferation, and clinicopathologic variables of the respective tumors.

Materials and Methods

Patients, pO_2 measurement, and tissue specimens. Archival tissue of cervical cancer samples with known intratumoral hypoxia levels was used for the analysis. The material was derived from a series of 48 consecutive patients for whom sufficient tumor material for analysis was available. These patients presented to the Department of Gynecology and Obstetrics at Leipzig University between 2001 and 2003 [International Federation of Gynecologists and Obstetricians (FIGO) stage IB-IV; Table 1] and prior to therapy underwent intratumoral oxygenation measurements with the Eppendorf histography system (Eppendorf, Hamburg, Germany) according to the standard procedure described earlier (31). The procedure was done after informed written consent was given by each patient. The study was approved by the medical ethics committee of Leipzig University. pO_2 measurement was done in the conscious patient along at least two distinct tracks within the macroscopically vital tumor. For each track, approximately 30 data points were collected starting at a tissue depth of 5 mm. To confirm that the measurement was done within the tumor and not in necrotic or tumor-free areas, a needle core biopsy of ~2 mm in diameter and 20 mm in length was taken of each measured track after the procedure. The biopsies were formalin-fixed and paraffin-embedded according to standard protocols, followed by an evaluation by a gynecologic pathologist. A correlation analysis between the median pO_2 of each track and Epo and EpoR protein expression in the corresponding biopsy was done (see below).

Immunohistochemical staining for Epo, EpoR, and Ki-67. Immunohistochemical staining was done according to standard procedures. The sections were stained with rabbit polyclonal anti-Epo and anti-EpoR antibodies (Epo: H-162, sc-7956; EpoR: C-20, sc-695; both from Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal anti-Ki-67 antibody (clone Ki-S5; DAKO, Carpinteria, CA).

Briefly, after blocking of endogenous peroxidase and tissue avidin and biotin (DAKO), slides were incubated with the anti-Epo antibody (dilution, 1:100) overnight at 4°C, followed by incubation with a biotinylated anti-rabbit secondary antibody (Dako CSA Rabbit Link) and the CSA system from DAKO. Staining was visualized by using 3,3′-diaminobenzidine chromogen (DAKO). For EpoR, slides were incubated with the anti-EpoR antibody (dilution, 1:250) overnight at 4°C, followed by incubation with a biotinylated Pan-specific antibody (horse biotinylated anti-mouse/rabbit/goat IgG; Vector Laboratories, Burlingame, CA) and the Vectastain Elite ABC system (Vector Laboratories). Staining was visualized by using 3,3′-diaminobenzidine chromogen. Negative controls were done by omitting the respective antibodies in the primary antibody incubation. For Epo and EpoR, slides of adult kidney (32) and placenta (33) were used as positive controls.

For Ki-67, slides were boiled in target retrieval solution (DAKO) for 30 minutes in a pressure cooker for antigen demasking, and incubated overnight with the anti-Ki-67 antibody (dilution, 1:50) at 4°C. This was followed by incubation with a biotinylated secondary antibody (link anti-mouse antibody, DAKO) and the CSA system from DAKO. Staining was visualized by using 3,3′-diaminobenzidine chromogen.

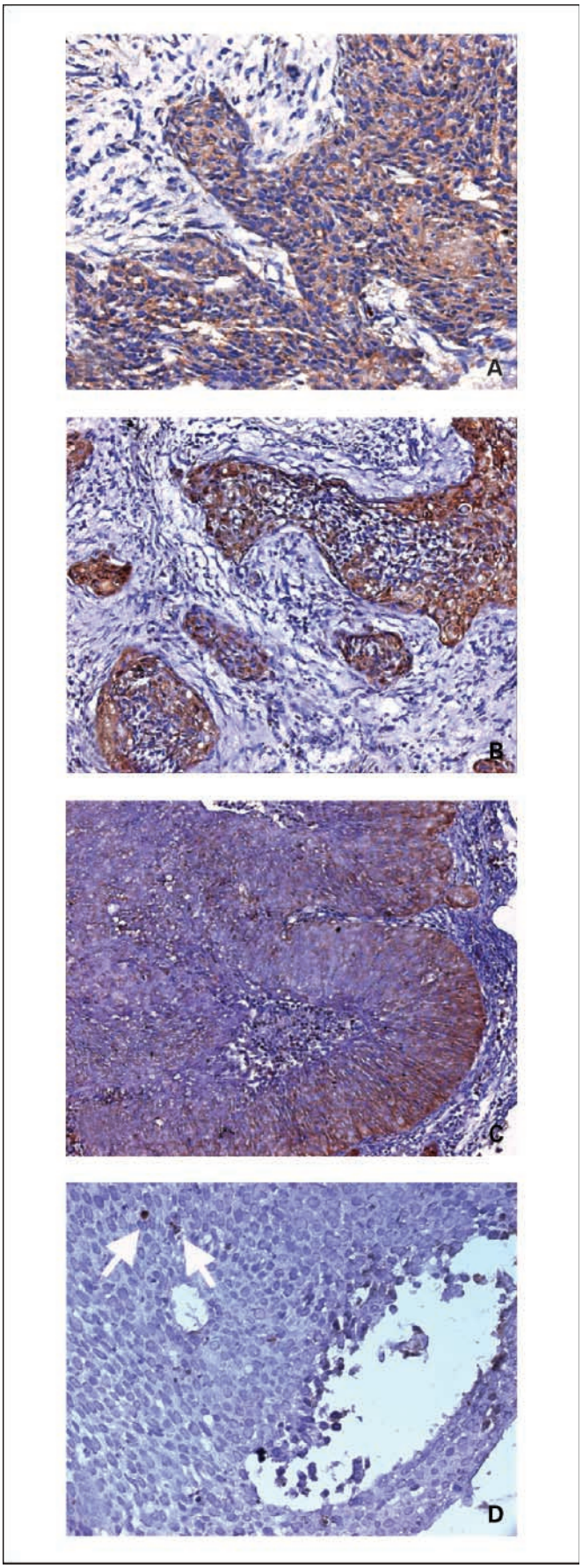
Table 1. Patient and tumor characteristics at the time of pretherapeutic pO_2 measurements

	No. of patients
FIGO stage	
I	12
II	17
III	13
IV	6
Grade	
1	8
2	27
3	13
pT stage	
pT_{1b1}	15
pT_{1b2}	2
pT_{2b}	2
pT_4	1
n.a.	28
pN stage	
N_0	16
N_1	4
n.a.	28
Lymphovascular space involvement	
L0	15
L1	33
Tumor diameter (mm)	
Median (range)	44 (17-100)
Patient age (y)	
Median (range)	47 (24-79)
Treatment modality	
Radical hysterectomy with pelvic ± paraaortic lymph node dissection	19
Primary exenteration	1
Radiation therapy	28
Tumor oxygenation pO_2 (mm Hg)	
Median (range)	8.6 (0.8-33.3)
≤10 mm Hg	26
>10 mm Hg	22

Abbreviations: pT stage, pathologic tumor stage; pN stage, pathologic node stage; n.a., not applicable (treated by radiation therapy).

Terminal deoxynucleotidyl transferase–mediated deoxyuracil triphosphate nick-end labeling assays. Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuracil triphosphate (dUTP) nick-end labeling (TUNEL). Slides were treated with the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI) according to the instructions of the manufacturer. Briefly, after routine deparaffinization, sections were digested with proteinase K (20 mg/mL) for 5 minutes at 37°C and incubated with the reaction mixture (1:100) for 60 minutes at 37°C. This was followed by incubation with a streptavidin-peroxidase complex (1:500) for 30 minutes at room temperature and subsequent color development with 3,3′-diaminobenzidine. As positive controls, DNase-treated lymph node sections were used, and for negative controls, the terminal deoxynucleotidyl transferase enzyme was omitted.

Evaluation of immunostaining. For the evaluation of cytoplasmic staining results for Epo and EpoR, a predefined scoring system based on the product of staining intensity and the percentage of positive tumor cells was used (21). Staining intensity was evaluated as negative (0), weak (1), moderate (2), strong (3), and the percentage of positive tumor cells was categorized as follows: (0) 0%, (1) 1% to 10%, (2) 11% to 50%, (3) 51% to 80%, and (4) > 80%. By multiplying both components, an expression score (0-12) was obtained. This score was



used for the correlation analyses. Evaluation of the samples was done by two independent investigators who were blinded to the patient data. In cases of discrepant assessment, an agreement was obtained after collegial revision using a multiheaded microscope.

To assess the effect of Epo and EpoR on survival, the Epo and EpoR expression scores were divided into high and low scores using the median expression score for Epo and EpoR, respectively, as the cutpoint (21).

Cells with clear brown nuclear labeling were defined as Ki-67- or TUNEL–positive, respectively. For the Ki-67 labeling index, 1,000 tumor cells were counted under 400× magnification, and the rate of Ki-67 positive cells was calculated as a percentage. Analogously, to determine the apoptotic index (AI) of a tumor, the number of terminal deoxynucleotidyl transferase–mediated deoxyuracil triphosphate nick-end labeling–positive cells in 1,000 tumor cells was expressed as a percentage.

Statistical analysis. To evaluate the association between ordinal data, the Spearman correlation coefficient was calculated, and for categorical data, χ^2 test was used. Groups were compared by use of Kruskal-Wallis *H* test and Mann-Whitney *U* test. Overall survival, with deaths due to any cause as event, and relapse-free survival, with relapse and metastases as events, were analyzed by log rank test. Kaplan-Meier curves and 3-year survival rates with 95% confidence intervals (95% CI) are presented. Cox regression analysis was done to assess the effect of Epo on overall survival as adjusted for FIGO stage and treatment modality. FIGO stage was dichotomized into early (FIGO I-II) and advanced (FIGO III-IV). The estimator of the effect is expressed as relative risk with 95% CI and corresponding *P* values.

$P < 0.05$ were considered to indicate statistical significance. Statistical analysis was done using the statistics package SPSS (version 11.5 for Windows; SPSS GmbH, Munich, Germany).

Results

Patient characteristics and clinicopathologic features. All cervical carcinomas were clinically staged according to FIGO criteria. The median age at diagnosis was 47 years (range, 24-79 years). In 19 of the examined cases, the tumor was resected by total mesometrial resection (34) along with pelvic/para-aortic lymph node dissection. In one case (FIGO IV), the tumor was treated with curative intent by primary laterally extended endopelvic exenteration (35). For these patients, the tumors were additionally staged according to the pTNM system. Twenty-eight patients were treated by radiation therapy. The distribution of FIGO and TNM stages is shown in Table 1. Forty-one tumors were of squamous cell origin, six cancers represented adenocarcinomas, and one was an adenosquamous cell carcinoma. The median hemoglobin level at the time of biopsy was 8.1 mmol/L (range, 5.6-9.6 mmol/L). For two patients, the hemoglobin levels were not available.

Expression of Epo and EpoR protein in cervical cancers. Immunohistochemistry was done in all 48 cervical cancer samples. Epo protein expression was observed in 88% of the investigated cases. Positive tumor cells presented a diffuse, cytoplasmic staining (Fig. 1A). In 25% of cancers, Epo expression was accentuated at

Fig. 1. *A,* diffuse cytoplasmic staining for Epo in squamous cell carcinoma of the uterine cervix (original magnification, ×214). *B,* strong cytoplasmic staining for EpoR in squamous cell carcinoma of the uterine cervix (original magnification, ×214). *C,* pronounced staining for EpoR at the front of invasion in squamous cell carcinoma of the uterine cervix (original magnification, ×150). *D,* dark stained cells, terminal deoxynucleotidyl transferase – mediated deoxyuracil triphosphate nick-end labeling – positive cells in cervical cancer (*arrows*).

the infiltrating edge of the respective tumor. Proximity to necrosis did not influence the Epo expression pattern (data not shown).

For EpoR, cytoplasmic immunostaining was present in 92% of cervical cancers (Fig. 1B). In 38% of all samples, EpoR expression was more pronounced at the infiltrating edge of tumors (Fig. 1C). Adjacent necroses did not have an influence on EpoR expression (data not shown). There was a significant positive correlation between Epo and EpoR protein expression in the investigated tumors (r = 0.36, P = 0.013) and in 81% of the cancers, coexpression of Epo and EpoR was found.

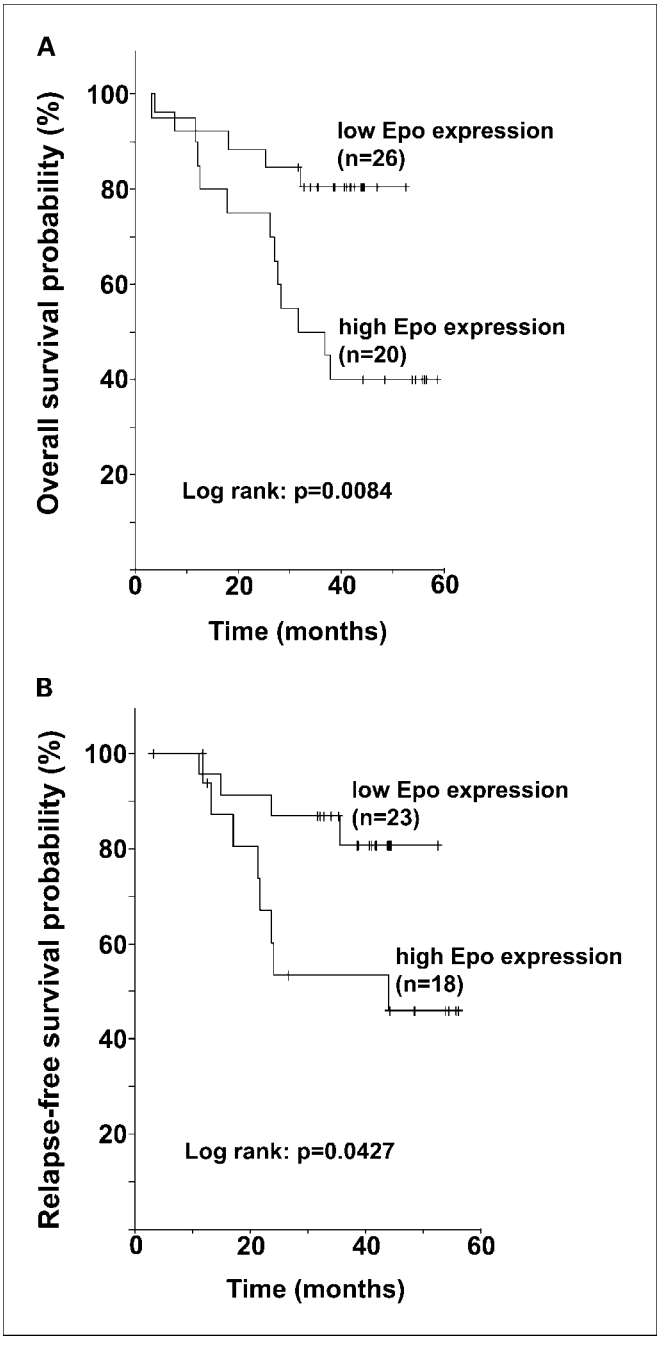


Fig. 2. Kaplan-Meier analysis showing that high Epo expression significantly correlates with reduced overall survival (*A*) and reduced relapse-free survival (*B*) in patients with cervical cancer.

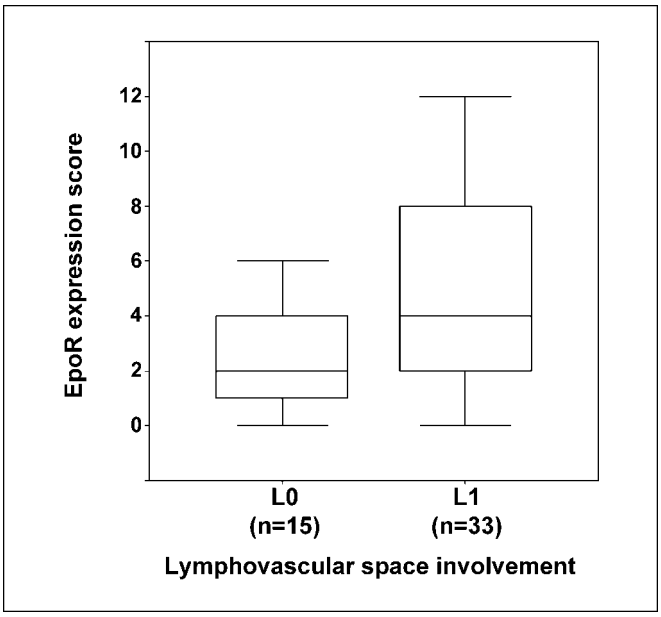


Fig. 3. EpoR expression in cervical cancers without (L0) and with (L1) lymphovascular space involvement.

Epo and EpoR expression and intratumoral pO_2. For the 48 tumors, the median oxygenation along the histologically confirmed single tracks used for Epo and EpoR immunostaining was 8.9 mm Hg (range, 0.8-33.3 mm Hg). There was no correlation between the intratumoral oxygenation and Epo or EpoR expression, respectively (Epo: $r = -0.08$, $P = 0.59$; EpoR: $r = 0.24$, $P = 0.096$). In tumors with a median pO_2 >10 mm Hg, the EpoR expression localized significantly more often with the infiltrating edge of the tumor (57% versus 27%, P = 0.047), whereas no such association could be found for Epo.

Epo and EpoR expression and clinical outcome. The median follow-up period was 44 months (95% CI, 41.4-46.8). For two patients, no follow-up data were available for survival analysis. Five patients (four with disease progression and one case with unknown relapse status) were not included in the analysis for relapse-free survival. As described in Materials and Methods, the median expression score for Epo (median = 2) and EpoR (median = 2) was used to compare low-expressing and high-expressing groups for survival. To analyze the effect of Epo on overall survival, univariate and multivariate Cox regression models were calculated. Patients with a high Epo expression score had a significantly reduced overall survival [3-year rate: 50.0% (95% CI, 28.1-71.9%) versus 80.6% (95% CI, 65.3-95.9%); P = 0.008; Fig. 2A] and a significantly reduced relapse-free survival [3-year rate: 53.6% (95% CI, 28.3-78.8%) versus 80.8% (95% CI, 63.4-98.1%); P = 0.043; Fig. 2B]. Epo had an independent significant effect on overall survival after adjustment for FIGO stage and treatment modality (relative risk, 3.0; 95% CI, 1.0-8.8%; P = 0.047). Hemoglobin levels had no relevant clinical effect in univariate or multivariate analyses, nor did they influence the observed effect of Epo on survival.

For EpoR, there was no significant difference in overall survival and relapse-free survival comparing the high EpoR expression and low EpoR expression groups (overall survival,

3-year rate: 56.5% versus 78.2%, $P = 0.136$; relapse-free survival, 3-year rate: 68.8% versus 70.3%; $P = 0.719$).

Epo and EpoR expression and clinicopathologic variables. Tumors with lymphovascular space involvement showed significantly higher EpoR scores ($P = 0.037$; Fig. 3). Furthermore, there was a significant positive correlation between EpoR expression and tumor size ($r = 0.32$, $P = 0.032$). We observed no correlation between Epo expression and lymphovascular space involvement or tumor diameter. Also, there was no association between Epo/EpoR expression and histologic grade, FIGO stage, lymph node status, histology type, or hemoglobin level (Table 2).

Epo and EpoR expression, proliferation, and apoptosis. The AI and Ki-67 labeling index were determined in 40 of the 48 cervical carcinomas. The remaining eight cases were excluded because no sufficient material from the needle core biopsies was left for analyses. The median AI was found to be 1.2% (range, 0.3-3.4%; Fig. 1D), whereas the median Ki-67 labeling index was 36.6% (range, 8.5-75.8%). There was a significant positive correlation between Epo expression and AI ($r = 0.49$, $P = 0.001$l Fig. 4A) and between EpoR expression and AI ($r = 0.36$, $P = 0.021$; Fig. 4B). We found no correlation between Epo or EpoR expression and the Ki-67 labeling index ($r = -0.13$; $P = 0.43$; $r = -0.14$, $P = 0.39$).

Discussion

To our knowledge, this is the first study analyzing the effect of Epo and EpoR expression on the survival of patients with cervical cancer. Furthermore, the relationship of Epo and EpoR expression to intratumoral pO_2 levels and apoptosis in cervical cancer was investigated.

In our study, we found Epo expression in 88% and EpoR expression in 92% of cases. This is in line with findings by Acs et al. who showed Epo expression in 14 out of 15 cervical cancers, and showed EpoR expression in all investigated samples (11). In our cohort, cervical cancers with high Epo expression resulted in a significantly reduced overall and relapse-free survival, whereas no statistically significant survival difference was observed for high EpoR expression. This finding implies that Epo-expressing cervical cancers are more aggressive.

Recently, Epo and its receptor were shown in a variety of other solid tumors including head and neck cancer (19, 21), breast cancer (10, 36), non–small cell lung cancer (20), and endometrial cancer (17). Hypoxia is a feature of many solid tumors and may render a malignant tumor more aggressive (37). Although a great number of genes have been shown to be hypoxia-inducible *in vitro*, there is a paucity of studies investigating hypoxia-induced gene and protein expression in tumors with quantified pO_2 levels. Therefore, the association between intratumoral hypoxia and the expression of hypoxia-related markers is still not well defined (8). In our study, we compared Epo and EpoR expression to the degree of intratumoral hypoxia of the respective cervical cancers. To minimize the potential for sampling errors, needle core biopsies were collected directly following invasive oxygenation measurement. With this method, we were able to

Table 2. Association between clinicopathologic variables and Epo/EpoR expression

Clinicopathologic variables	Epo score median (min-max)	*P*	EpoR score median (min-max)	*P*
Histologic grade				
1	1 (0-6)	0.130*	1.5 (0-9)	0.134*
2	4 (0-12)		4 (0-12)	
3	2 (0-12)		2 (1-6)	
FIGO stage				
I	2 (0-6)	0.511*	1.5 (0-6)	0.063*
II	2 (0-12)		6 (0-12)	
III	2 (0-12)		2 (0-12)	
IV	2.5 (0-6)		4 (1-9)	
pN stage				
pN_0	2 (0-6)	0.601†	2 (0-9)	0.900†
pN_1	1 (0-9)		2 (2-2)	
Lymphovascular space involvement				
L0	2 (1-12)	0.897†	2 (0-6)	0.037†
L1	2 (0-12)		4 (0-12)	
Histology				
Squamous cell carcinoma	2 (0-12)	0.993*	2 (0-12)	0.222
Adenocarcinoma + adenosquamous cell carcinoma	2 (1-9)		2 (0-6)	

Clinicopathologic variables	Epo correlation coefficient (Spearman)	*P*	EpoR correlation coefficient (Spearman)	*P*
Hemoglobin level	0.034	0.832‡	0.071	0.639‡
AI	0.486	0.001‡	0.363	0.021‡
Ki-67 index	−0.129	0.427‡	−0.139	0.391‡
Tumor size	0.137	0.370‡	0.32	0.032‡

* Kruskal-Wallis *H* test was used to analyze the association.
† Mann-Whitney *U* test was used to analyze the association.
‡ Spearman rank correlation was used to analyze the association.

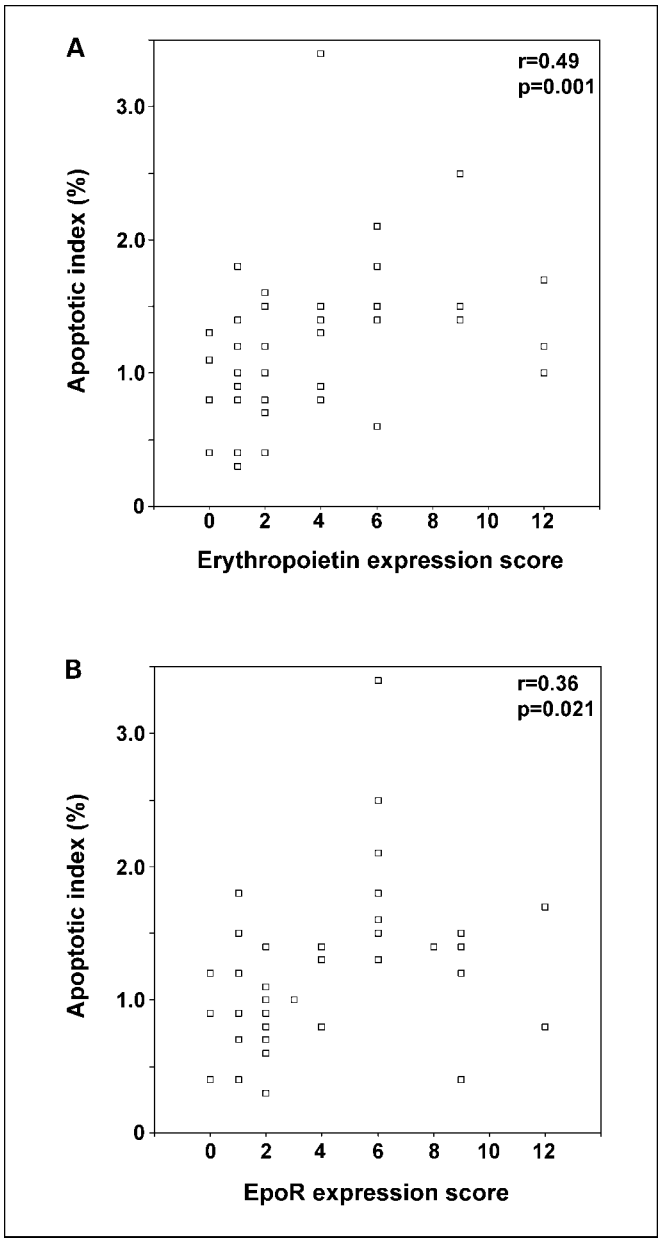


Fig. 4. *A,* positive correlation of immunohistochemical expression of Epo with the AI in cervical cancers. *B,* positive correlation of immunohistochemical expression of EpoR with the AI in cervical cancers.

perform the immunohistochemical analysis on tissue samples with a defined pO_2 value, allowing a precise description of the spatial relationship between intratumoral oxygenation and the respective protein expression. In our study, there was no significant correlation between EpoR expression and intratumoral oxygenation values, suggesting hypoxia-independent mechanisms of EpoR induction *in vivo*. However, in well-oxygenated cervical cancers with a median pO_2 of >10 mm Hg (the commonly used threshold for hypoxic cervical cancers; ref. 1), EpoR expression localized significantly more often to the infiltrating edge of tumors, an area thought to belong to the most hypoxic parts of solid cancers (38). This finding is therefore in agreement with the previously described hypoxia-inducible EpoR signaling in cervical cancer cell lines *in vitro* (11). In our samples, we did not observe a correlation between Epo expression and tumor hypoxia. Physiologically, Epo is up-regulated by Hif-1, the most sensitive and specific transcription factor under hypoxic conditions (39). A recent study by Winter et al. in head and neck squamous cell carcinoma showed a significant correlation between Epo and Hif-1 expression (21). The observed lack of a direct correlation between Epo expression and tumor hypoxia in our samples might be explained by the fact that Hif-1 is not only induced by hypoxia, but also by a variety of other stimuli, including tumor suppressor inactivation and oncogene activation (40). Furthermore, our observation is consistent with another clinical study in cervical cancer that showed no association between Hif-1 and intratumoral oxygenation measured with the Eppendorf electrode (30). Additionally, a recent report by Arcasoy et al. investigated Epo expression and tumor hypoxia determined by pimonidazole binding in head and neck squamous cell cancer (19). The authors showed that Epo expression did not always colocalize with regional tumor hypoxia as determined by pimonidazole binding. Another previous study in breast cancer also did not show consistent colocalization of Epo expression and hypoxia as determined by pimonidazole binding (36). These data suggest additional mechanisms of Epo induction in tumor cells.

In our cohort of cervical cancers, we found a significant positive correlation between the AI and Epo/EpoR expression, respectively. Our findings have several possible interpretations: first, Epo/EpoR pathways may not be functional in the investigated cervical cancers or may have a biological role that differs from their antiapoptotic and proliferative effects in hematopoiesis (13). Second, even in the presence of functional pathways, the Epo/EpoR system may fail to prevent apoptosis, e.g., because of alterations in sequence, structure, secretion, or subcellular localization of its components. Although evidence for an autocrine-paracrine influence of endogenous Epo on tumor cells has previously been reported (36, 41, 42), a recent *in vivo* study found no influence of recombinant human Epo on tumor growth, proliferation rate, and tumor angiogenesis (43). Third, the Epo/EpoR system might be up-regulated to compensate for the high apoptosis rates observed in a subset of our investigated cervical cancers and, subsequently, mediate antiapoptotic effects. This hypothesis is consistent with the observed reduced survival of patients with high Epo-expressing cervical cancers in our study. Additionally, this hypothesis could explain the negative effects of recombinant human Epo on patient outcome in head and neck as well as breast cancers (23, 24) that were documented in two large clinical trials, as exogenous Epo might further propagate malignant progression. Because other reports as well as a recent meta-analysis did not find an unfavorable effect on overall survival of the treated cancer patients (25), future functional studies will have to further characterize the role of the Epo/EpoR system in malignant tumors.

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