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Expression of Epidermal Growth Factor Receptor (EGFR) and Activated EGFR Predict Poor Response to (Chemo)radiation and Survival in Cervical Cancer

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Abstract

Purpose: Activation of the epidermal growth factor receptor (EGFR) signaling pathway has been reported to induce resistance to (chemo)radiation in cancers, such as head and neck cancer, whereas EGFR-targeted agents in combination with (chemo)radiation seem to improve treatment efficacy. The aim of this study was to determine the relation between proteins involved in the EGFR pathway and response to (chemo)radiation and survival in a large, well-documented series of cervical cancer patients.

Experimental Design: Pretreatment tissue samples of 375 consecutive International Federation of Gynecologists and Obstetricians stage Ib to IVa cervical cancer patients treated with (chemo)radiation between January 1980 and December 2006 were collected. Clinicopathologic and follow-up data were prospectively obtained during standard treatment and follow-up. Protein expression of EGFR, phosphorylated EGFR (pEGFR), PTEN, phosphorylated AKT, and phosphorylated extracellular signal-regulated kinase (pERK) was assessed by immunohistochemistry on tissue microarrays.

Results: EGFR staining was present in 35.3%, pEGFR in 19.7%, PTEN in 34.1%, phosphorylated AKT in 4.1%, and pERK in 29.2% of tumors. pEGFR staining was related to PTEN ($P = 0.001$) and pERK staining ($P = 0.004$). EGFR staining was inversely related to PTEN ($P = 0.011$). In multivariate analysis, membranous staining of EGFR [hazard ratio (HR), 1.84; 95% confidence interval (95% CI), 1.20-2.82; $P = 0.005$] and cytoplasmic staining of pEGFR (HR, 1.71; 95% CI, 1.11-2.66; $P = 0.016$) were independent predictors of poor response to (chemo)radiation. Membranous EGFR staining also was an independent prognostic factor for poor disease-specific survival (HR, 1.54; 95% CI, 1.09-2.17; $P = 0.014$).

Conclusions: EGFR and pEGFR immunostainings are frequently observed and independently associated with poor response to therapy and disease-specific survival in cervical cancer patients primarily treated by (chemo)radiation. Our data present the EGFR pathway as a promising therapeutic target in already ongoing clinical trials. (Clin Cancer Res 2009;15(23):7389–97)

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Standard treatment of locally advanced cervical cancer has changed from radiotherapy alone to concurrent platinum-based chemoradiation. Despite this change, the 5-year survival in patients with locally advanced cervical cancer is still ~52% (1). Currently, there are no (biological) markers available that accurately predict response to (chemo)radiation.

Epidermal growth factor receptor (EGFR) is involved in the ErbB signaling pathway, which is often dysregulated in cancer. Autophosphorylation of EGFR to phosphorylated EGFR (pEGFR) leads to activation of two downstream pathways: the Ras/Raf/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase/ERK pathway and the phosphatidylinositol 3-kinase/AKT pathway. *PTEN* (*phosphatase and tensin homologue deleted on*

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Translational Relevance

Although clinical trials where epidermal growth factor receptor (EGFR) inhibitors are added to standard chemoradiation in cervical cancer patients have already been started, up to now the prognostic significance of EGFR protein expression in cervical cancer was unclear. The aim of our study was to determine the relation between EGFR and its pathway members and response to (chemo)radiation and survival in a large, well-documented consecutive series of locally advanced-stage cervical cancer patients treated with (chemo)radiation. Our study indicates that EGFR protein expression predicts poor response to (chemo)radiation and worse disease-specific survival and presents the EGFR pathway as a promising therapeutic target in already ongoing clinical trials.

chromosome 10) acts as a tumor suppressor gene by inhibiting phosphorylation and thereby activation of AKT (2, 3). Both downstream EGFR pathways are involved in processes associated with carcinogenesis and tumor progression, such as inhibition of apoptosis, cell migration, cell growth, and angiogenesis (4), and more recently also in conferring resistance to irradiation (5, 6).

EGFR and some of its downstream targets have been studied previously in cervical cancer, but conflicting results about their prognostic significance have been reported (7–12). Expression of phosphorylated AKT (pAKT) in cervical cancer seemed to be related to local recurrence as a measure for radiation resistance (13), and Faried et al. (14) showed that patients with pAKT-negative tumors had a more favorable prognosis. In contrast, Lee et al. (15) found an inverse correlation for pAKT with survival. Hypermethylation and mutations of the *PTEN* gene have been associated with poor outcome after radiotherapy (16, 17). Thus, the prognostic significance of different components of the EGFR pathway in cervical cancer is equivocal due to small various (frequently a mix of primarily surgically and radiotherapeutically treated) patient populations and differences in immunohistochemistry. Moreover, protein expression of EGFR and its downstream targets have not been studied before in relation to response to (chemo)radiation in cervical cancer.

In head and neck squamous cell carcinoma, protein expression of EGFR seems to be related to a higher local relapse rate, indicating a poor response to radiotherapy (18, 19). Moreover, a recent randomized clinical trial showed a significant prolonged progression-free survival for head and neck squamous cell cancer patients treated with radiotherapy in combination with cetuximab, a chimeric human mouse anti-EGFR monoclonal antibody, when compared with standard radiotherapy (20).

The aim of the present study was to determine protein expression of EGFR, pEGFR, PTEN, pAKT, and pERK in relation to response to (chemo)radiation and survival in a large, well-documented series of cervical cancer patients.

Materials and Methods

Patients. For the present study, all patients primarily treated by radiotherapy or chemoradiation in the University Medical Center Groningen or in collaborating hospitals between January 1980 and December 2006 were selected. Patients with stage IVb disease were excluded, as their treatment was individualized. Follow-up data were collected for at least 5 y or up to January 2008. Staging was done according to International Federation of Gynecologists and Obstetricians guidelines. Radiotherapy included external beam radiotherapy up to 45 Gy and low-dose rate brachytherapy, two applications of 17.5 Gy. Concurrent chemotherapy before 1999 consisted of three cycles of carboplatin and 5-fluorouracil. Carboplatin dose was 300 mg/m^2, dissolved in 250 mL 5% glucose, given over 30 min i.v. on day 1. 5-Fluorouracil dose was 600 mg/m^2, dissolved in 2 L of saline and administered i.v. continuously on days 2 to 5. This cycle was repeated another two times every 28 d. After 1999, chemotherapy consisted of 40 mg/m^2 cisplatin i.v. once a week for 6 wk concomitant with external pelvic and intracavitary radiation. Paraffin-embedded, formalin-fixed primary tumor tissue was collected from each patient. Patients were only included in the analysis if enough tumor tissue was available for tissue microarray (TMA) construction.

Institutional Review Board approval. In the University Medical Center Groningen clinicopathologic and follow-up, data are prospectively obtained during standard treatment and follow-up and stored in a computerized registration database. For the present study, all relevant data were retrieved from this computerized database into a separate, anonymous database. Patient identity was protected by study-specific, unique patient numbers. Codes were only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathologic and follow-up data, the larger databases could only be checked through the data managers, thereby ascertaining the protection of patients' identity. Using the registration database, all tissue specimens were identified by unique patient numbers and retrieved from the archives of the Department of Pathology. Therefore, according to Dutch law, no further Institutional Review Board approval was needed.[6]

Evaluation of response to (chemo)radiation. In the period up to 1993, eligible patients underwent an additive hysterectomy 6 to 8 wk after completion of (chemo)radiation. After 1993, only patients with residual disease in a biopsy taken 8 to 10 wk after completion of primary treatment underwent surgery (21). Hysterectomy or biopsy after (chemo)radiation was only done when a patient (technically) was judged to be operable. As post-(chemo)radiation biopsy and/or hysterectomy to evaluate response to (chemo)radiation) were done in only a selected group of patients, response to (chemo)radiation was evaluated retrospectively in two models. Model I: Response to (chemo)radiation was determined by locoregional disease-free survival in all patients, which was defined as the period from diagnosis to clinical locoregional progression of disease during treatment or to locoregional recurrence after treatment. If location of recurrence was unknown, patients were not included in this analysis. Model II: To be able to analyze two populations with supposedly the highest difference in sensitivity to (chemo)radiation, two populations with optimal and very poor response to (chemo)radiation were defined. Patients with complete disease eradication were patients with no residual disease in their post-treatment biopsy/hysterectomy specimen and who did not have a locoregional recurrence in the follow-up, with a follow-up time of at least 2 y, versus patients with clinical evidence of disease progression during treatment or clinical evidence of disease persistence at examination after completion of primary treatment.

TMA construction. For the construction of the TMA, only pretreatment biopsies were used. Areas of representative tumor tissue were marked on H&E-stained slides of the paraffin-embedded tissue. Areas of necrosis and areas with severe leukocyte infiltration were avoided. The TMAs were constructed by using a precision instrument (Beecher Instruments). Three cores of 0.6 mm in diameter were punched from the marked area on the paraffin-embedded tissue (donor block). These

[6] http://www.federa.org

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cores were then placed in a blank paraffin block (recipient block) in predefined locations. After inserting all the cores, the recipient block was placed in an oven at 37°C for 2 min to attach the cores to the surrounding paraffin. Each TMA also contained internal controls, including healthy tissue (skin epithelia, normal cervical tissue, and colon polyps) and tumor tissue (breast, colon, and ovarian cancer). In total, seven TMAs, each containing ~200 cores, were constructed.

Immunohistochemistry. For immunohistochemistry, 3-μm sections were cut from the TMA. These sections were mounted on aminopropyl-ethoxy-silan–coated glass slides (Sigma-Aldrich). Details of the antibodies used for immunohistochemistry and methods for antigen retrieval are summarized in Supplementary Table S1. For antibody detection, the avidin-biotin-peroxidase method was used for all, except pAKT. For pAKT staining, the EnVision horseradish peroxidase system (Dako) was used. Slides were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxidase for 30 min. Staining was visualized by 3,3′-diaminobenzidine and counterstaining was done with hematoxylin.

Evaluation of staining. Staining intensity was semiquantitatively scored as negative (0), weak positive (1+), positive (2+), and strong positive (3+). In addition, the percentage of positive cells was recorded. In case of differences between cores, scores were averaged for statistical analyses. Tumors were considered positive for EGFR in case of ≥10% membranous staining (22). pAKT and pERK stainings were considered as positive if >10% of tumor cells showed positive (2+) cytoplasmic and/or nuclear staining (23). Positive staining of PTEN was defined as >10% cytoplasmic staining (16). Positive pEGFR staining was defined as at least weak positive (1+) cytoplasmic staining, as the activated EGFR is internalized (24). Scoring was done by two independent observers (M.G.N. and K.A.H.) without knowledge of clinical data. A concordance of >90% for all stainings was found. The discordant cases were reviewed and scores were reassigned on consensus of opinion. Only patients with at least two representative cores were included in the analysis.

Statistical analysis. Statistical analysis was done with Statistical Package for the Social Sciences 16.0 for Windows (SPSS, Inc.). Differences in age were compared with the Student's t test. Other baseline characteristics, as well as associations between stainings, were compared with the Pearson's χ^2 test. Associations between the presence of positive immunostaining and clinicopathologic characteristics were assessed in logistic regression models, where immunostaining was used as dependent factor and the clinicopathologic characteristics were used as independent factors. To determine factors involved in the presence of poor response to (chemo)radiation (model II), response to (chemo)radiation (as dependent factor) was evaluated in relation to clinicopathologic factors and protein expression (as independent factors) with logistic regression analysis. Because treatment modality (radiotherapy versus chemoradiation) is not a patient/tumor-dependent factor but a time-dependent factor, as standard treatment changed over time from radiotherapy alone to chemoradiation, it was included in multivariate analysis. Factors with a P value of >0.10 were excluded stepwise in multivariate analysis; in the final step, only factors with a P value of <0.05 were included. Disease-specific survival (DSS) was defined as the period from diagnosis to death as a consequence of cervical cancer or last follow-up visit alive or death from another cause. Overall survival (OS) was defined as the time from diagnosis to death of any cause or last follow-up visit alive. Survival curves were generated using the Kaplan-Meier method, with evaluation of the differences by the Mantel-Cox log-rank test. Differences in locoregional disease-free survival (model I), DSS, and OS according to clinicopathologic characteristics and protein expression were analyzed using Cox regression analyses. As chemoradiation is a time-dependent factor, multivariate analyses were adjusted for treatment modality. Variables with a P value of >0.10 in univariate analysis were excluded stepwise in multivariate analysis; in the final step, only factors with a P value of <0.05 were included. P values of <0.05 were considered statistically significant.

Results

Patient and tumor characteristics. From January 1980 to December 2006, 489 patients were diagnosed with cervical cancer and primarily treated with (chemo)radiation. In 375 cases (77%), sufficient pretreatment tissue was available for TMA construction. The baseline characteristics of the 114 patients from whom no tumor tissue could be obtained differed from the study population, as they had more often advanced-stage (≥IIb) disease ($P < 0.001$). The other baseline characteristics were comparable (data not shown). Clinicopathologic characteristics of patients included in this study are summarized in Table 1. Median follow-up time was 3.4 years (range, 0.1-18.3) for all patients. For patients still alive at last follow-up, median follow-up time was 6.0 years (range, 0.5-18.3). Primary radiotherapy was given to 189 (50%) patients, whereas (chemo) radiation was given to 186 (50%) patients. The only difference in baseline characteristics between these two groups was that patients primarily treated with chemoradiation were younger (median age, 46.8 versus 64.8; $P < 0.001$). Biopsies taken 8 to 10 weeks after completion of primary treatment or hysterectomy specimens of 279 of 375 (74%) patients were available to evaluate response to therapy. The patients who did not underwent biopsy or hysterectomy after therapy were significantly older than patients who did (median age, 69.8 versus 50.6; $P < 0.001$).

Clinicopathologic factors in relation to staining of EGFR, pEGFR, and pAKT. Immunohistochemistry was done for

Table 1. Patient and tumor characteristics

	***n* = 375**
Age at diagnosis	
Median	54
Range	21-92
	n (%)
FIGO stage	
Ib1	42 (11%)
Ib2	27 (7%)
IIa	51 (14%)
IIb	179 (48%)
IIIa	11 (3%)
IIIb	51 (14%)
IVa	14 (4%)
Histology	
Squamous	311 (83%)
Adenocarcinoma	52 (14%)
Other	12 (3%)
Differentiation grade	
Good/moderate	223 (59%)
Poor	128 (34%)
Unknown	24 (6%)
Lymphangioinvasion	
No	248 (66%)
Yes	54 (14%)
Unknown	73 (19%)
Tumor diameter	
0-4 cm	99 (26%)
≥4 cm	238 (63%)
Unknown	38 (10%)

Abbreviation: FIGO, International Federation of Gynecologists and Obstetricians.

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EGFR, pEGFR, PTEN, pAKT, and pERK. The proportion of patients with less than two representative tissue cores varied from 1.6% to 5.1%. Supplementary Fig. S1 shows a representative negative and positive tumor for each staining. Healthy cervical epithelium showed weak positive membranous EGFR and cytoplasmic pEGFR expression. PTEN stained positive and pERK stained weakly positive in the cytoplasm as well as in the nuclei, whereas pAKT was negative in cervical epithelium.

Positive EGFR staining was present in 129 of 365 (35.3%), positive pEGFR staining in 71 of 361 (19.7%), positive PTEN staining in 126 of 369 (34.1%), positive pERK staining in 104 of 356 (29.2%), and positive pAKT staining in 15 of 364 (4.1%) of tumors. pEGFR staining was positively related to PTEN ($P = 0.001$) and pERK staining ($P = 0.004$). EGFR positivity was inversely related to PTEN ($P = 0.011$). No other associations were found (data not shown).

Table 2. Relation between immunostaining and clinicopathologic factors

	EGFR$^-$, *n*/total (%)	**EGFR$^+$, *n*/total (%)**	**EGFR positive**	
			OR (95% CI)	***P***
Age (continuous)			1.00 (0.99-1.02)	0.717
Age ≥54	121/236 (51%)	66/129 (51%)		
Stage ≥IIb	151/236 (64%)	95/129 (74%)	1.57 (0.98-2.52)	0.061
Adenocarcinoma	46/226 (20%)	6/127 (5%)	0.19 (0.08-0.47)	<0.001
Poor differentiation	87/226 (38%)	39/117 (33%)	0.80 (0.50-1.28)	0.348
Lymphangioinvasion	30/189 (16%)	23/105 (22%)	1.49 (0.81-2.72)	0.199
Tumor diameter ≥4 cm	141/209 (67%)	90/119 (76%)	1.50 (0.90-2.49)	0.120
	pEGFR$^-$, *n*/total (%)	**PEGFR$^+$, *n*/total (%)**	**pEGFR positive**	
			OR (95% CI)	***P***
Age (continuous)			1.02 (1.00-1.03)	0.071
Age ≥54	144/290 (50%)	42/71 (59%)		
Stage ≥IIb	189/290 (65%)	56/71 (79%)	2.00 (1.07-3.70)	0.029
Adenocarcinoma	38/279 (14%)	14/71 (20%)	1.56 (0.79-3.07)	0.200
Poor differentiation	108/271 (40%)	14/68 (21%)	0.39 (0.21-0.74)	0.004
Lymphangioinvasion	50/234 (21%)	3/59 (5%)	0.20 (0.06-0.66)	0.008
Tumor diameter ≥4 cm	182/263 (69%)	46/61 (75%)	1.36 (0.72-2.59)	0.340
	PTEN$^-$, *n*/total (%)	**PTEN$^+$, *n*/total (%)**	**PTEN positive**	
			OR (95% CI)	***P***
Age (continuous)			1.01 (1.00-1.03)	0.085
Age ≥54	121/243 (50%)	69/126 (55%)		
Stage ≥IIb	168/243 (69%)	82/126 (65%)	0.83 (0.53-1.31)	0.429
Adenocarcinoma	33/237 (14%)	19/120 (16%)	1.16 (0.63-2.15)	0.629
Poor differentiation	82/229 (36%)	44/117 (38%)	1.00 (0.52-1.91)	1.000
Lymphangioinvasion	35/202 (17%)	17/96 (18%)	1.03 (0.54-1.94)	0.935
Tumor diameter ≥4 cm	160/225 (71%)	74/107 (69%)	0.91 (0.55-1.50)	0.716
	pAKT$^-$, *n*/total (%)	**pAKT$^+$, *n*/total (%)**	**pAKT positive**	
			OR (95% CI)	***P***
Age (continuous)			1.04 (1.00-1.08)	0.027
Age ≥54	175/349 (50%)	12/15 (80%)		
Stage ≥IIb	233/349 (67%)	12/15 (80%)	1.99 (0.55-7.20)	0.293
Adenocarcinoma	50/337 (15%)	0/15 (0%)	0.00 (0.00-0.00)	0.997
Poor differentiation	120/331 (36%)	3/11 (27%)	0.66 (0.17-2.53)	0.544
Lymphangioinvasion	49/282 (17%)	2/12 (17%)	0.95 (0.20-4.48)	0.949
Tumor diameter ≥4 cm	96/219 (44%)	12/13 (92%)	5.26 (0.67-41.03)	0.113
	pERK$^-$, *n*/total (%)	**pERK$^+$, *n*/total (%)**	**pERK positive**	
			OR (95% CI)	***P***
Age (continuous)			1.01 (0.99-1.02)	0.357
Age ≥54	129/252 (51%)	54/104 (52%)		
Stage ≥IIb	170/252 (67%)	67/104 (64%)	0.87 (0.54-1.41)	0.581
Adenocarcinoma	34/241 (14%)	16/103 (16%)	1.12 (0.59-2.13)	0.731
Poor differentiation	89/236 (38%)	36/97 (37%)	0.97 (0.60-1.59)	0.918
Lymphangioinvasion	35/199 (18%)	16/87 (18%)	1.06 (0.55-2.03)	0.870
Tumor diameter ≥4 cm	159/233 (68%)	67/89 (75%)	1.42 (0.81-2.47)	0.218

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Table 3. Response to (chemo)radiation

A	Model I (n = 364)	Univariate		Multivariate*	
		HR (95% CI)	*P*	HR (95% CI)	*P*
	Age	1.01 (0.99-1.02)	0.268	†	
	Stage ≥IIb	2.54 (1.51-4.29)	<0.001	2.66 (1.52-4.66)	0.001
	Adenocarcinoma	1.72 (1.06-2.79)	0.027	2.07 (1.24-3.44)	0.005
	Poor differentiation	1.04 (0.69-1.57)	0.861	†	
	Lymphangioinvasion	0.95 (0.53-1.69)	0.859	†	
	Tumor diameter ≥4 cm	2.15 (1.27-3.64)	0.004	‡	
	EGFR positive	1.77 (1.19-2.63)	0.005	1.84 (1.20-2.82)	0.005
	pEGFR positive	2.06 (1.35-3.15)	0.001	1.71 (1.11-2.66)	0.016
	PTEN positive	0.73 (0.47-1.13)	0.162	†	
	pAKT positive	0.67 (0.21-2.11)	0.495	†	
	pERK positive	1.14 (0.74-1.74)	0.552	†	
B	**Model II (n = 192)**	**Univariate**		**Multivariate***	
		OR (95% CI)	***P***	**OR (95% CI)**	***P***
	Age	1.01 (0.99-1.04)	0.241	†	
	Stage ≥IIb	2.93 (1.27-6.64)	0.011	‡	
	Adenocarcinoma	4.48 (1.83-10.98)	0.001	8.96 (2.99-26.85)	<0.001
	Poor differentiation	0.86 (0.41-1.77)	0.678	†	
	Lymphangioinvasion	1.64 (0.70-3.88)	0.258	†	
	Tumor diameter ≥4 cm	2.95 (0.16-7.53)	0.024	‡	
	EGFR positive	3.28 (1.63-6.61)	0.001	6.08 (2.39-15.47)	<0.001
	pEGFR positive	2.86 (1.31-6.22)	0.008	4.06 (1.58-10.43)	0.004
	PTEN positive	1.13 (0.56-2.26)	0.731	†	
	pAKT positive	0.51 (0.06-4.33)	0.535	†	
	pERK positive	1.13 (0.52-2.43)	0.759	†	

NOTE: Model I: Cox regression analysis for time to clinical locoregional progression of disease during treatment or to locoregional recurrence after treatment (A). Model II: logistic regression analysis for patients with clinical progression or persistence of disease after treatment versus patients with complete disease eradication (B).
*Adjusted for treatment modality.
†Not included in multivariate analysis.
‡Not included in the final step of the multivariate analysis.

Positive EGFR staining was less frequently observed in adenocarcinoma than in squamous cell carcinoma [odds ratio (OR), 0.19; 95% confidence interval (95% CI), 0.08-0.47; $P < 0.001$; Table 2]. pEGFR positivity was related to high tumor stage (≥IIb; OR, 2.00; 95% CI, 1.07-3.70; $P = 0.029$), whereas poor differentiation (OR, 0.39; 95% CI, 0.21-0.75; $P = 0.004$) and lymphangioinvasion (OR, 0.20; 95% CI, 0.06-0.66; $P = 0.008$) were less frequently observed in pEGFR-positive patients. Positive pAKT staining increased with age (OR, 1.04; 95% CI, 1.00-1.08; $P = 0.027$). No associations were found for PTEN and pERK staining and any clinicopathologic characteristic. As our specimens were collected over a long time period, we also analyzed, for instance, EGFR expression in carcinomas of the patients diagnosed before 1997 versus those after 1997 (in 1997, the formula of formalin was slightly changed). We found no significant differences between the frequency of positivity before (59 of 163) and after (70 of 202) 1997, indicating that expression is not affected by the storage time of the tissue blocks in this study.

EGFR and pEGFR are associated with response to (chemo)radiation. Locoregional progression during treatment or locoregional recurrence in follow-up was observed in 100 of 364 (27%) patients (model I). The location of recurrence of 11 patients was unknown, and therefore, these patients were not included in the analysis. Furthermore, 45 patients with clinical progression or persistence of disease at examination after completion of primary treatment and 147 patients with complete disease eradication were identified (model II). Forty-four of 45 patients with clinical progression or persistence of disease indeed died of their residual locoregional disease. Table 3 summarizes the relation between response to (chemo)radiation, immunohistochemical staining of the five parameters, and clinicopathologic characteristics in the two models. Univariate analysis revealed that EGFR and pEGFR stainings were related to poor response in both models, and therefore, these stainings were included in multivariate analysis. In model I, positive stainings of EGFR and pEGFR were independent predictors of poor response to therapy [EGFR: hazard ratio (HR), 1.84; 95% CI, 1.20-2.82; $P = 0.005$; pEGFR: HR, 1.71; 95% CI, 1.11-2.66; $P = 0.016$], as confirmed by an even stronger relation between EGFR and pEGFR and response in model II analyzing the most extreme groups with respect to response only. Furthermore, simultaneous positive staining of both EGFR and pEGFR ($n = 21$) was also significantly associated with response to (chemo)radiation in both univariate models (data not shown).

Positive EGFR staining is related to poor prognosis. Positive immunostaining of EGFR and pEGFR was also related to DSS and OS in univariate analysis (Table 4). During the follow-up period, 195 of 375 (52%) patients died, of which 151 died

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Table 4. Results of Cox regression analysis for disease-specific death and death from any cause

Disease-specific death (n = 375)	Univariate		Multivariate*	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	1.00 (0.99-1.01)	0.596	†	
Stage ≥IIb	2.35 (1.56-3.54)	<0.001	2.56 (1.67-3.93)	<0.001
Adenocarcinoma	1.54 (1.02-2.32)	0.040	1.71 (1.11-2.63)	0.014
Poor differentiation	1.24 (0.89-1.72)	0.211	†	
Lymphangioinvasion	1.10 (0.70-1.72)	0.675	†	
Tumor diameter ≥4 cm	2.06 (1.35-3.13)	0.001	‡	
EGFR positive	1.50 (1.08-2.08)	0.015	1.54 (1.09-2.17)	0.014
pEGFR positive	1.51 (1.04-2.20)	0.032	‡	
PTEN positive	0.80 (0.57-1.14)	0.222	†	
pAKT positive	0.59 (0.22-1.58)	0.293	†	
pERK positive	1.18 (0.83-1.68)	0.350	†	
Death from any cause (n = 375)	**Univariate**		**Multivariate***	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age	1.01 (1.01-1.02)	0.002	‡	
Stage ≥IIb	1.97 (1.39-2.78)	<0.001	1.97 (1.39-2.78)	<0.001
Adenocarcinoma	1.21 (0.81-1.79)	0.354	†	
Poor differentiation	1.15 (0.85-1.55)	0.355	†	
Lymphangioinvasion	1.12 (0.75-1.66)	0.590	†	
Tumor diameter ≥4 cm	1.69 (1.19-2.39)	0.003	‡	
EGFR positive	1.43 (1.07-1.91)	0.016	‡	
pEGFR positive	1.43 (1.02-2.00)	0.039	‡	
PTEN positive	0.87 (0.64-1.19)	0.383	†	
pAKT positive	0.71 (0.33-1.54)	0.390	†	
pERK positive	1.15 (0.83-1.58)	0.401	†	

*Adjusted for treatment modality.
†Not included in multivariate analysis.
‡Not included in the final step of the multivariate analysis.

of cervical cancer. The 5-year DSS rate was 53% in EGFR-positive patients versus 63% in EGFR-negative patients and 50% in pEGFR-positive patients versus 60% in pEGFR-negative patients (Fig. 1A and B). The 5-year OS rate was 47% in EGFR-positive patients versus 55% in EGFR-negative patients and 48% in pEGFR-positive patients versus 53% in pEGFR-negative patients (Fig. 1C and D). In multivariate Cox regression analysis for DSS including stage and histology, positive EGFR staining was independently related to poor DSS (HR, 1.54; 95% CI, 1.09-2.17; P = 0.014; Table 4). The relation between EGFR and OS was borderline significant (HR, 1.33; 95% CI, 0.99-1.77; P = 0.058), and therefore, EGFR was finally excluded from the stepwise multivariate analysis. pEGFR was not related to DSS (HR, 1.30; 95% CI, 0.86-1.98; P = 0.216) and OS (HR, 1.15; 95% CI, 0.78-1.68; P = 0.447) in multivariate analysis. Finally, simultaneous positive staining of both EGFR and pEGFR (n = 21) was also significantly associated with DSS and OS in univariate analysis (data not shown).

Discussion

Our study in a large, well-documented series of consecutive cervical cancer patients primarily treated with (chemo)radiation reveals that EGFR immunostaining is associated with poor DSS (HR, 1.54; 95% CI, 1.09-2.17; P = 0.014). Furthermore, this study is the first to report that positive immunostainings of EGFR (HR, 1.84; 95% CI, 1.20-2.82; P = 0.005) and pEGFR (HR, 1.71; 95% CI, 1.11-2.66; P = 0.016) predict poor response to (chemo)radiation in cervical cancer, independent of stage, histology, and treatment modality. In our study, response to (chemo)radiation was defined in two different ways. The relation of both EGFR (OR, 6.08; 95% CI, 2.39-15.47; P < 0.001) and pEGFR (OR, 4.06; 95% CI, 1.58-10.43; P = 0.004) with response was the strongest in our model with the highest contrast in radiosensitivity (model II), supporting the idea that indeed EGFR and pEGFR are associated with a poor response to (chemo)radiation in cervical cancer. Interestingly, in this model, stage was not an independent prognostic factor for poor response, whereas it is a strong prognostic factor for survival. However, response to (chemo)radiation is a different phenomenon not necessarily related to stage but to a variety of (cell biological) factors, such as hypoxia, but also EGFR and pEGFR expression. An increased staining of EGFR has also been shown to be involved in poor response to radiotherapy in other malignancies, for example, head and neck squamous cell cancer (18, 19). The relation between EGFR and response to radiotherapy might be explained by the fact that EGFR is involved in DNA double-strand break repair (25). Radiation-induced EGFR signaling activates the phosphatidylinositol 3-kinase/AKT pathway, resulting in DNA double-strand break repair by interaction with DNA-dependent protein kinases (26). Another explanation might be that radiation activates EGFR signaling even in the absence of ligand binding, for example,

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by increasing transforming growth factor-α expression, which can activate EGFR (27). As a consequence, this activation of the downstream signaling cascades causes inhibition of apoptosis and promotion of cell proliferation (4). Therefore, carcinomas with increased levels of EGFR or pEGFR might activate this EGFR signaling pathway more efficiently, resulting in a decreased local control.

Because of its apparent involvement in response to radiotherapy, EGFR-targeted therapy has recently been implemented as a new therapeutic strategy in various malignancies (reviewed in ref. 28). However, the relation between EGFR protein expression and response to EGFR inhibitors is questionable, as colorectal cancer patients without detectable EGFR protein expression did respond to treatment with cetuximab (29, 30). Various EGFR-related biomarkers were found to predict response to treatment with EGFR inhibitors. For instance, in colorectal cancer and non–small cell lung cancer, *KRAS* mutations are associated with resistance to EGFR inhibitors (31), whereas specific *EGFR* mutations and high copy numbers of the *EGFR* gene predict a better response in non–small cell lung cancer (32, 33). In cervical cancer, neither *EGFR* mutations (34) nor *EGFR* gene amplification (35) and only a few (0-8%) *KRAS* mutations (36–39) have been observed. These data combined with the relation that we find for EGFR and pEGFR immunostaining and poor response to (chemo)radiation suggest that the addition of EGFR inhibitors to standard chemoradiation should be evaluated in advanced-stage cervical cancer patients. Up to now, in cervical cancer, only a single phase II study using gefitinib, an EGFR tyrosine kinase inhibitor, as monotherapy for recurrent cervical cancer was reported recently with modest response rates (40). Clinical trials with cetuximab in addition to (chemo)

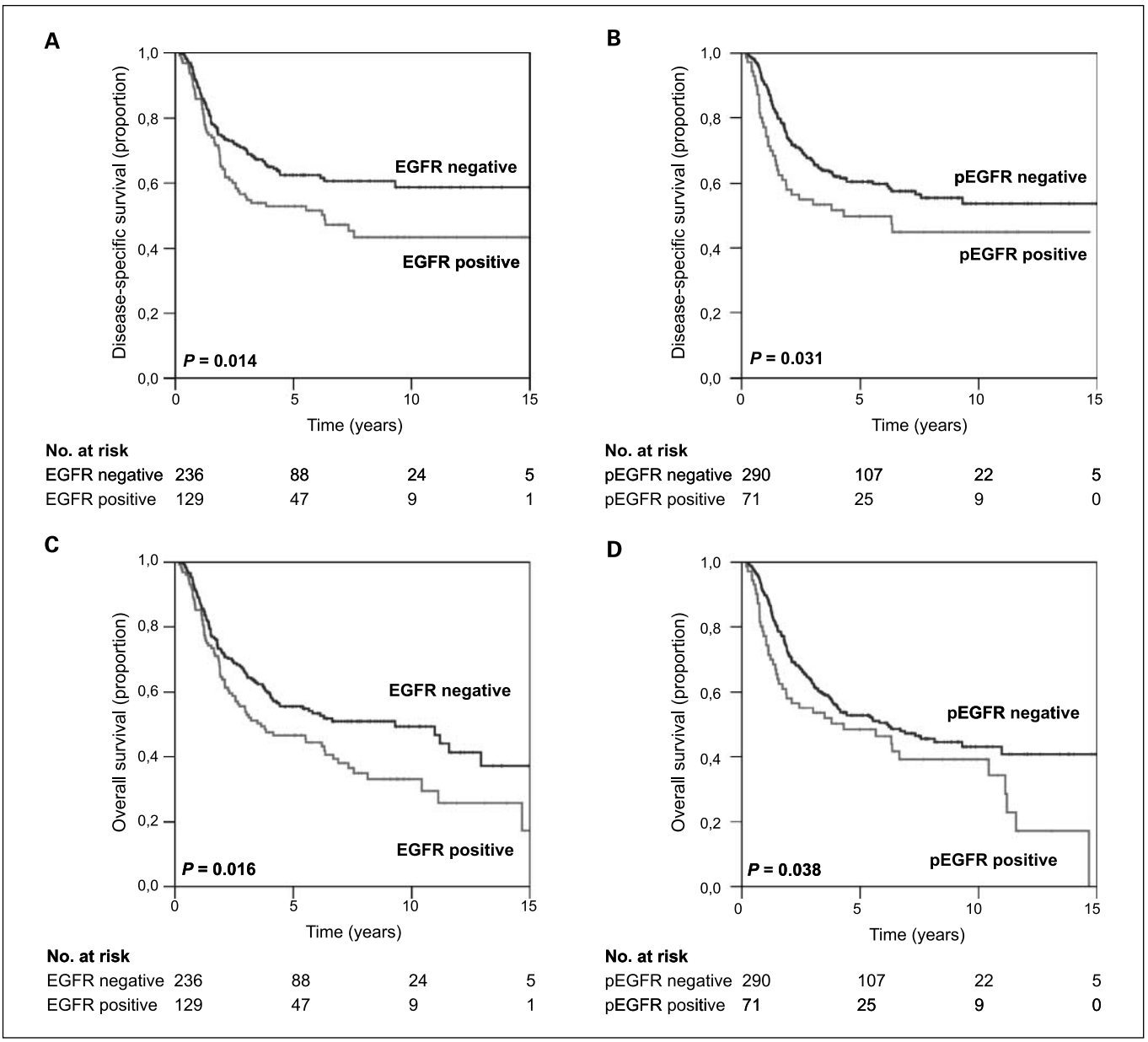

Fig. 1. Survival curves. Kaplan-Meier curves for the relation of EGFR and pEGFR immunostaining with DSS (*A* and *B*) and OS (*C* and *D*).

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radiation in the treatment of locally advanced cervical cancer are ongoing.[7]

No relation was found between PTEN, pAKT, and pERK and response to (chemo)radiation. Positive PTEN staining was observed in 34.1% of tumors, which is lower than previously reported (16, 41, 42). This might be due to differences in study populations, as other studies mainly focused on early-stage cervical cancer and positive PTEN staining decreases in more advanced-stage disease (16). In our study, pAKT was only positive in 4.1% of tumors. In previous studies, pAKT immunostaining was observed in 29% to 94% (13, 14, 43), although the same antibody and protocol for immunostaining were used. pAKT staining was not related to response to therapy nor to survival possibly due to the relatively small number of positive cases in our study. To our knowledge, this is the first study investigating pERK protein expression in cervical cancer. Activated ERK was not related to response to therapy nor to survival.

In conclusion, our study indicates that EGFR and pEGFR immunostainings are independent markers for poor response to (chemo)radiation and EGFR immunostaining is an independent poor prognostic factor for DSS. In advanced-stage cervical cancer patients, the apparent involvement of EGFR in response to (chemo)radiation presents the EGFR pathway as a promising therapeutic target in already ongoing clinical trials, in which EGFR inhibitors are combined with standard chemoradiation in cervical cancer patients.

[7] http://www.cancer.gov/clinicaltrials, NCT00104910.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Expression of Epidermal Growth Factor Receptor (EGFR) and Activated EGFR Predict Poor Response to (Chemo)radiation and Survival in Cervical Cancer

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