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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

Membranous Expression of Ectodomain Isoforms of the Epidermal Growth Factor Receptor Predicts Outcome after Chemoradiotherapy of Lymph Node–Negative Cervical Cancer

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Abstract

Purpose: We compared the prognostic significance of ectodomain isoforms of the epidermal growth factor receptor (EGFR), which lack the tyrosine kinase (TK) domain, with that of the full-length receptor and its autophosphorylation status in cervical cancers treated with conventional chemoradiotherapy.

Experimental Design: Expression of EGFR isoforms was assessed by immunohistochemistry in a prospectively collected cohort of 178 patients with squamous cell cervical carcinoma, and their detection was confirmed with Western blotting and reverse transcriptase PCR. A proximity ligation immunohistochemistry assay was used to assess EGFR-specific autophosphorylation. Pathways associated with the expression of ectodomain isoforms were studied by gene expression analysis with Illumina beadarrays in 110 patients and validated in an independent cohort of 41 patients.

Results: Membranous expression of ectodomain isoforms alone, without the coexpression of the full-length receptor, showed correlations to poor clinical outcome that were highly significant for lymph node–negative patients (locoregional control, $P = 0.0002$; progression-free survival, $P < 0.0001$; disease-specific survival, $P = 0.005$ in the log-rank test) and independent of clinical variables. The ectodomain isoforms were primarily 60-kD products of alternative EGFR transcripts. Their membranous expression correlated with transcriptional regulation of oncogenic pathways including activation of *MYC* and *MAX*, which was significantly associated with poor outcome. This aggressive phenotype of ectodomain EGFR expressing tumors was confirmed in the independent cohort. Neither total nor full-length EGFR protein level, or autophosphorylation status, showed prognostic significance.

Conclusion: Membranous expression of ectodomain EGFR isoforms, and not TK activation, predicts poor outcome after chemoradiotherapy for patients with lymph node–negative cervical cancer. *Clin Cancer Res; 17(16); 5501–12. ©2011 AACR.*

Introduction

The epidermal growth factor receptor (EGFR) is frequently overexpressed in locally advanced cervical cancers (1, 2). There is a need to improve the conventional chemoradiotherapy of these patients (3), and adjuvant therapy with EGFR inhibitors has been proposed (4, 5). Clinical trials are underway to assess monoclonal antibodies against the extracellular domain (ECD) of the receptor and intracellular inhibitors of the tyrosine kinase (TK) activity (Clinical Trials: http://www.cancer.gov). The importance of EGFR and its TK activation for disease progression is, however, not clear; studies relating receptor level to clinical outcome show inconsistent results (1, 2, 6–10). Moreover, studies on other tumor types have failed to show a correlation between EGFR phosphorylation and clinical outcome, as well as in cases when the EGFR level was prognostic (11, 12). Recent research has suggested that EGFR can exert tumorigenic functions through TK independent pathways in addition to TK activation (13). These findings indicate possible mechanisms of the resistance to EGFR-targeted therapies and should be considered when the importance of the receptor is explored in clinical studies (14).

The full-length EGFR (isoform A) is composed of an ECD with a ligand binding site, a transmembrane domain (TMD), and an intracellular domain (ICD) with a number of phosphorylation sites (15). Several EGFR mutants exist, for which the constitutively activated EGFRvIII isoform with deletions in the ECD is the most common (16), whereas

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Translational Relevance

Membranous expression of ectodomain epidermal growth factor receptor (EGFR) isoforms may serve as a well needed biomarker for identifying lymph node–negative cervical cancer patients at risk of relapse after conventional chemoradiotherapy. The ectodomain EGFR isoforms might also be efficient targets for therapy. Adjuvant EGFR therapy, either targeting the extracellular domain (ECD) of the receptor or inhibiting its tyrosine kinase (TK) activity, is currently investigated in clinical trials on cervical cancers. Our results may shed light on possible mechanisms for treatment resistance in such trials and have implications for the choice of therapy. Hence, EGFR TK inhibitors will probably not have the desired therapeutic effect in the lymph node–negative patients in whom the TK activity seems to play a minor role for disease progression. Monoclonal antibodies against EGFR might interfere with a possible oncogenic function of the ectodomain isoforms and be more efficient.

mutations in the TK domain are rare in cervical cancers (17). Ligand binding or transactivation leads to receptor dimerization, phosphorylation, and TK activity. This initiates signaling cascades that may result in enhanced proliferation and metastatic potential (18). Ligand binding may also lead to proteolytic cleavage of the full-length receptor (19), and shedding of the cleavage products from the cell surface may indicate a malignant phenotype (20).

In addition to the full-length EGFR, 3 ectodomain EGFR isoforms (B, C, and D) without TK activity are generated from alternative transcripts encoding only the ECD (21). Although little is known about isoform B, the transcripts of isoforms C and D encodes protein products of 60/80 kD and 90/110 kD, respectively. These isoforms may have a tumor-suppressive function through ligand sequestering or interaction with the full-length receptor, inhibiting its activation (22, 23). However, oncogenic functions have also been shown for TK receptors lacking the TK domain (24, 25). Recently, a work by Weihua and colleagues (24) showed that EGFR may interact with and stabilize a membranous glucose transporter, thereby facilitating glucose uptake under energy-deprived conditions. The ICD was beneficial for this interaction, but not essential, and TK activation was not involved. EGFR interaction through the ECD with other membranous oncoproteins such as the mucin MUC1 has also been reported (26). It is possible that ectodomain EGFR isoforms could serve as a stabilizer and/or activator of these proteins in a similar way as the full-length receptor and thereby promote tumorigenic signaling. Although of utmost importance for the choice of adjuvant EGFR-targeted therapy, the clinical importance of ectodomain EGFR lacking the TK domain has not yet been clarified.

The present work was conducted to investigate the presence and prognostic significance of ectodomain EGFR isoforms, the full-length receptor, and its autophosphorylation status in a uniformly treated cohort of squamous cell cervical carcinomas receiving chemoradiotherapy as a primary treatment. Tumors expressing only ectodomain isoforms were separated from those expressing the full-length or all isoforms by immunohistochemistry, using 2 antibodies binding to the ICD and ECD of the receptor, respectively. The isoform type and possible transcript origin were explored in selected tumors with immunoblotting and reverse transcriptase PCR (RT-PCR). EGFR-specific assessment of the autophosphorylation status (Tyr1068) was achieved by the proximity ligation immunohistochemistry assay (PLA) on the basis of the simultaneous binding of antibodies against ICD and phosphorylated Tyr1068 (27), as described in our previous work (28). We further explored signaling pathways that were correlated with the expression of only ectodomain isoforms by gene expression analysis of 110 of the tumors and validated the results in an independent cohort of 41 tumors. Our study suggests that the membranous expression of ectodomain EGFR isoforms is associated with the activation of oncogenic pathways and is a strong prognostic factor in cervical cancers, whereas neither the full-length receptor nor its autophosphorylation status has any impact on the clinical outcome.

Patients and Methods

Patients and tumor specimens

One hundred ninety-one patients with primary squamous cell carcinoma of the uterine cervix, stage 1B bulky through 4A, were recruited to our chemoradiotherapy protocol at the Norwegian Radium Hospital from 1999 to 2006. Nineteen patients with adenocarcinoma, adenosquamous carcinoma, or small-cell undifferentiated carcinoma, who were enrolled during the same period, were not included in the study. Routine formalin-fixed and paraffin-embedded pretreatment tumor specimens were available in sufficient amounts for immunohistochemical study in 178 (93%) of the patients (Table 1). One to 4 snap-frozen tumor specimens, collected at the time of diagnosis and stored at −80°C, were available for Western blotting, RT-PCR, and gene expression profiling. An independent cohort of 41 patients with the same histology and recruited to the same chemoradiotherapy protocol until 2009 (Supplementary Table S1) was used to validate the results from the gene expression analysis. Formalin-fixed tumor specimens were available for 29 of these patients, all recruited after 2006. The study was approved by the regional committee of medical research ethics in southern Norway, and written informed consent was obtained from all patients.

Pathologic lymph nodes in the pelvis at the time of diagnosis were detected by MRI or, in a few cases, computed tomography. A lymph node was classified as pathologic whenever the short axis was equal to or exceeded 10 mm, according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 (29). All patients were treated with external irradiation and brachytherapy combined with cisplatin and followed up as described (30).

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Table 1. Patient and tumor characteristics by EGFR expression status

Characteristic	All patients		EGFR positive							
			Total EGFR		Full length EGFR		Ectodomain EGFR only		Phospho-EGFR	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Diagnostic information										
No. of patients	178		135		117		32		50	
Age, y										
Median	56		55		57		49		57	
Range	25–85		25–82		25–85		28–81		28–84	
FIGO stage										
1B	9	5	6	4	5	4	2	6	4	8
2	106	59	82	61	73	62	16	50	24	48
3	53	30	40	30	33	28	11	34	20	40
4A	10	6	7	5	6	5	3	9	2	4
Tumor volume,[a] cm^3										
Median	43.2		45.1		39.3		53.4		45.0	
Range	2.8–321.0		2.8–321.0		2.8–321.0		5.9–280.0		6.8–321.0	
Pelvic lymph node status										
Positive	73	41	58	43	46	39	16	50	21	42
Negative	105	59	77	57	71	60	16	50	29	58
Follow-up data										
Observation time,[b] mo										
Median	49		48		46		58		48	
Range	18–93		18–93		18–93		27–80		24–93	
Relapse										
Locoregional only	12	7	10	7	6	5	4	13	3	6
Distant only	39	22	28	21	19	16	12	38	11	22
Locoregional and distant	10	6	9	7	6	5	3	9	2	4
Cancer-related death	50	28	40	30	27	23	15	47	15	30

NOTE: Total EGFR, full-length EGFR, and ectodomain EGFR only were assessed in 178 patients, whereas phospho-EGFR was assessed in 97 patients who were positive for full-length EGFR.

Abbreviation: FIGO, Federation International de Gynecologie et d'Obstetrique.

[a]Determined from pretreatment magnetic resonance images and calculated on the basis of 3 orthogonal diameters (*a*, *b*, and *c*) as $(\pi/6)abc$.

[b]Based on patients without distant or locoregional relapse.

External radiation of 50 Gy was given to tumor and parametria and 45 Gy to the rest of the pelvis in 25 fractions. Endocavitary brachytherapy included 21 Gy in 5 fractions to point A. Adjuvant cisplatin (40 mg/m^2) was given weekly in maximum 6 courses during the period of external radiation. The follow-up included clinical examinations every third month for the first 2 years, twice a year for the next 3 years, and thereafter once a year. In cases of symptoms of recurrent disease, MRI of pelvis retroperitoneum and radiography of thorax were carried out. The time between diagnosis and the first event of relapse (progressive disease) or cancer-related death was recorded. Relapse was classified as locoregional (progression within the irradiated field in the pelvis), distant, or both. Endpoints were locoregional control (control within the irradiated pelvic volume including regional lymph nodes), progression-free survival (PFS, survival without locoregional and/or distant relapse), and disease-specific survival (DSS, not dead from cervical cancer). Ten patients died of causes not related to cervical cancer and were censored at the time of death.

Immunohistochemistry

Immunohistochemistry was carried out with the monoclonal mouse EGFR antibodies clone H11 (1:50; Dako Corp.), binding the ECD (aa 348–363; provided Dec. 15, 2009, by A. Enghøj, Dako Corp.), and clone EGFR.25 (1:150; Novocastra Laboratories Ltd.), binding the ICD outside the TK domain (http://www.leica-microsystems.com/fileadmin/img_uploads/novocastra_reagents/Novocastra_datasheets/egfr-384-ce.pdf; Fig. 1A and Supplementary Fig. S1). Staining with the ECD-antibody and the ICD-antibody reflected the total EGFR level and the full-length

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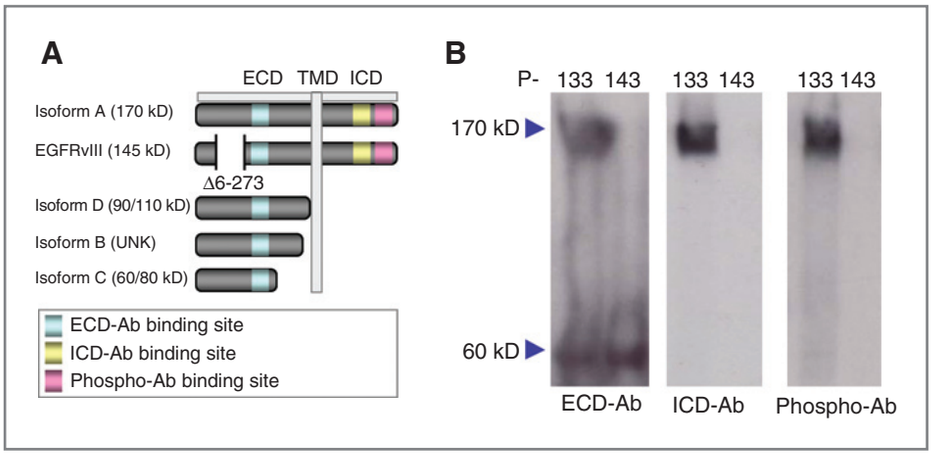

Figure 1. A, antibody (Ab) binding sites on the ECD and ICD on the most common isoforms of the EGFR, named according to the NCBI database. Three different antibodies were used: the ECD-Ab, ICD-Ab, and phospho-Ab. UNK, unknown size. B, Western blots showing detection of EGFR isoforms in 2 cervical tumors by the ECD-Ab, ICD-Ab, and phospho-Ab. The full-length and short isoforms were visualized with the ECD-Ab, full-length isoforms with the ICD-Ab, and full-length phosphorylated isoforms with the phospho-EGFR antibody. Expression of the full-length receptor, its phosphorylated form, and a short isoform of 60 kD is seen in P-133, whereas P-143 show expression of an ectodomain isoform of 60 kD only.

(isoform A and EGFRvIII) level, respectively, whereas positive staining with the ECD-antibody and negative staining with the ICD-antibody reflected expression of ectodomain isoforms without coexpression of the full-length receptor. Tissue sections (4 μm) were stained using the Dako EnVision +System, Peroxidase (DAB; K4007), and Autostainer (Dako Corp.). Antigen retrieval was done by microwaving the slides in 1 mmol/L EDTA (pH 8.0) for 15 minutes (ICD-antibody) or by treatment with proteinase (S3020; Dako Corp.) for 5 minutes (ECD-antibody). Samples were incubated overnight at 4°C. As negative controls, the antibodies were substituted with mouse myeloma protein of the same concentration and subclass as the monoclonal antibodies. A cervical tumor known to express EGFR served as a positive control. Nuclear, cytoplasmic, and membranous staining cases were scored on the basis of the percentage of positive tumor cells: 0, 0%; 1, 1%–10%; 2, 11%–50%; and 3, >50%, where tumors with a score of 2 or 3 were considered positive. All cases of immunohistochemical and PLA staining (described later) were scored by an experienced scientist at the Department of Pathology (R. Holm), who was blinded to the clinical outcome.

PLA

PLA (Duolink *in situ* PLATM; Olink Bioscience; ref. 27) with the mouse ICD-antibody (clone EGFR.25; 1:75) and the rabbit phospho-EGFR antibody #18-2463 (1:50; Zymed Laboratories; Fig. 1A and Supplementary Fig. S1) was used to detect EGFR-specific phosphorylation of Tyr1068 as a measure of the autophosphorylation status of the full-length receptor (including EGFRvIII) in 97 patients with ICD-antibody staining scores of 1 to 3 (28). With this method, staining occurred only when the phospho-EGFR antibody was bound in proximity to the ICD-antibody (27), ensuring specificity for EGFR. Antigen retrieval with EDTA was carried out as described earlier, and the samples were incubated overnight at 4°C with the 2 antibodies. The Duolink Detection Kit HRP/NovaRED with PLA plus and minus probes for mouse and rabbit (Olink Bioscience) was used to visualize the bound antibody pairs, according to the manufacturer's description. Specimens were mounted with the Duolink Brightfield Mounting Medium (Olink Bioscience). A cervical tumor known to express phosphorylated EGFR served as a positive control. The phospho-specificity of the staining was confirmed and presented in a previous work (28). The PLA staining was scored as described in the earlier section.

Western blotting

Western blotting was carried out in 2 tumors to confirm the detection of different EGFR isoforms by the various antibodies (Fig. 1B) and further to evaluate the presence of the various isoforms in 12 tumors, as described in the Results section. Frozen sections were treated with lysis buffer containing 1% NP-40, 10% glycerol, 20 mmol/L Tris HCL at pH 7.5, 137 mmol/L NaCl, 100 mmol/L NaF, 1 mmol/L sodium vanadate, 1 mmol/L PMSF, 0.02 mg/mL aprotinin, 40 μL/mL complete protease inhibitor cocktail (F. Hoffman-La Roche Ltd.), 10 μL/mL each of phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich), and dH_2O up to 2 mL. The lysates were vortexed, sonicated for 4 minutes, incubated on ice for 1 to 2 hours, and centrifuged. Supernatants containing 30 μg of protein were denatured at 95°C for 5 minutes, separated on 8% SDS-polyacrylamide gels (Thermo Scientific), blotted on polyvinylidene difluoride membranes (Millipore Corp.), and stained with the primary EGFR antibody of interest and either goat anti-rabbit (P0448; Dako Corp.) or donkey anti-mouse (715-001-003; Jackson ImmunoResearch Laboratories Inc.) secondary antibody. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) chemiluminescence kit was used for detection.

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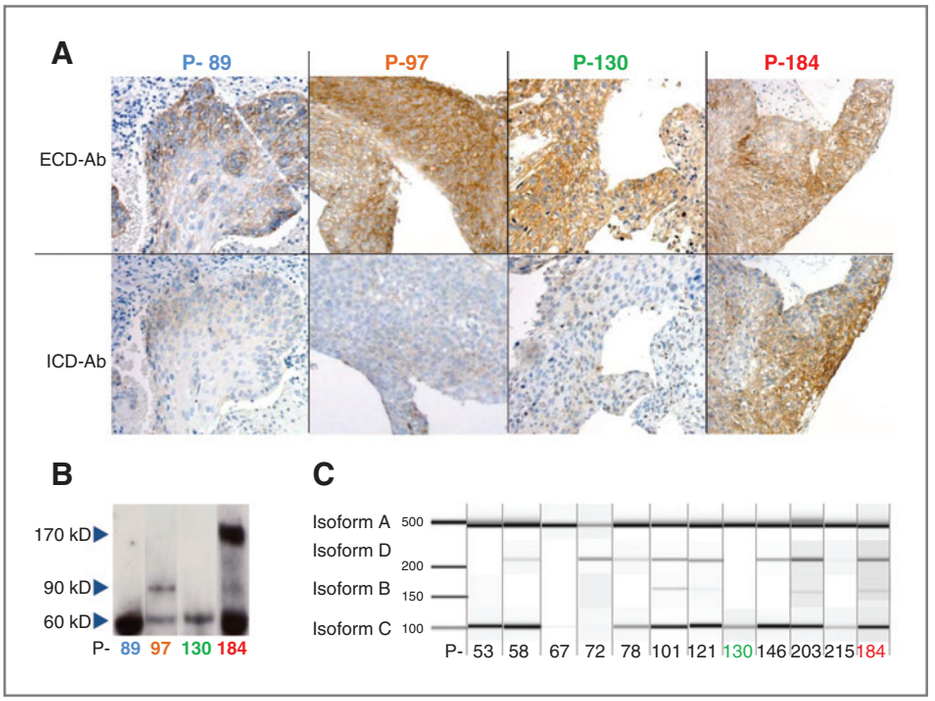

Figure 2. A, tumor sections from cervical tumors with expression of only ectodomain isoforms in the membrane (P-89, P-97, P-130), visualized by positive ECD-antibody (Ab) and negative ICD-Ab membranous staining, and a cervical tumor with coexpression of the full-length receptor and ectodomain isoforms (P-184), as shown by positive staining with both antibodies. B, Western blots confirming the expression of only ectodomain isoforms in tumors with only ectodomain isoforms in the membrane by immunohistochemistry (P-89, P-97, P-130) and the coexpression of ectodomain isoforms with the full-length receptor in P-184. C, detection of EGFR isoforms A, B, C, and D by RT-PCR in 11 tumors with only ectodomain isoforms in the membrane by immunohistochemistry and in 1 tumor coexpressing the EGFR isoforms by immunohistochemistry (P-184). A and B, the immunohistochemistry and Western data of P-130 and P-184.

Reverse transcriptase PCR

RT-PCR was carried out to identify possible EGFR isoform (A, B, C, and D) transcripts in 18 tumors. Total RNA was isolated from the frozen specimens by using TRIzol reagent (Invitrogen; ref. 30). Several specimens from the same tumor were pooled. Satisfactory RNA quality was ensured by the use of the Agilent 2100 Bioanalyzer (Agilent Technologies). Superscript III transcriptase (Invitrogen) was used to synthesize cDNA (10 ng) from the total RNA. RT-PCR was carried out using the HotStarTaq Master Mix (Quiagen, Inc). Primers for EGFR isoform A were designed toward the ICD of EGFR, whereas primers for isoforms B to D were as listed in Albitar and colleagues (ref. 31 and Supplementary Table S2). Totally 500 ng of cDNA was added as a template to the master mix together with the appropriate primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. A number of 35 PCR cycles with an annealing temperature of 59°C were carried out. The products were detected by the use of the Agilent DNA 1000 Kit (Agilent Technologies)

Gene expression profiling

Gene expression profiling was done to identify pathways associated with the expression of ectodomain isoforms. The frozen specimens of 110 patients in the basic cohort and all 41 patients in the validation cohort had more than 50% tumor cells in hematoxylin and eosin–stained sections, derived from the central part of the biopsy, and were included in the analysis. Illumina beadarrays human WG-6 v3 (Illumina Inc.) with 48,000 transcripts were used as reported (32). Total RNA was synthesized as described earlier. Illumina TotalPrep RNA amplification kit (Ambion Inc.) was used to amplify RNA, using 500 ng of total RNA as the input material. cRNA was synthesized overnight, labeled, and hybridized to the arrays at 58°C overnight. The hybridized arrays were stained with streptavidin-Cy3 (PA43001; Amersham TM) and scanned with an Illumina beadarray reader. The Genome Studio software (Illumina Inc.) was used for signal extraction and quality control, and quantile normalization was done in J-Express (MolMine AS). Satisfactory quality of the arrays and samples was observed in all cases. The Illumina data have been deposited to the GEO repository (GSE29817).

Statistics

Correlations involving protein data were investigated using the Spearman's rank correlation analysis. Kaplan–Meier curves were compared using the log-rank test. Uni- and multivariate Cox proportional hazard analyses were used to evaluate prognostic parameters with respect to clinical endpoints. *P* values of less than 0.05 were considered significant.

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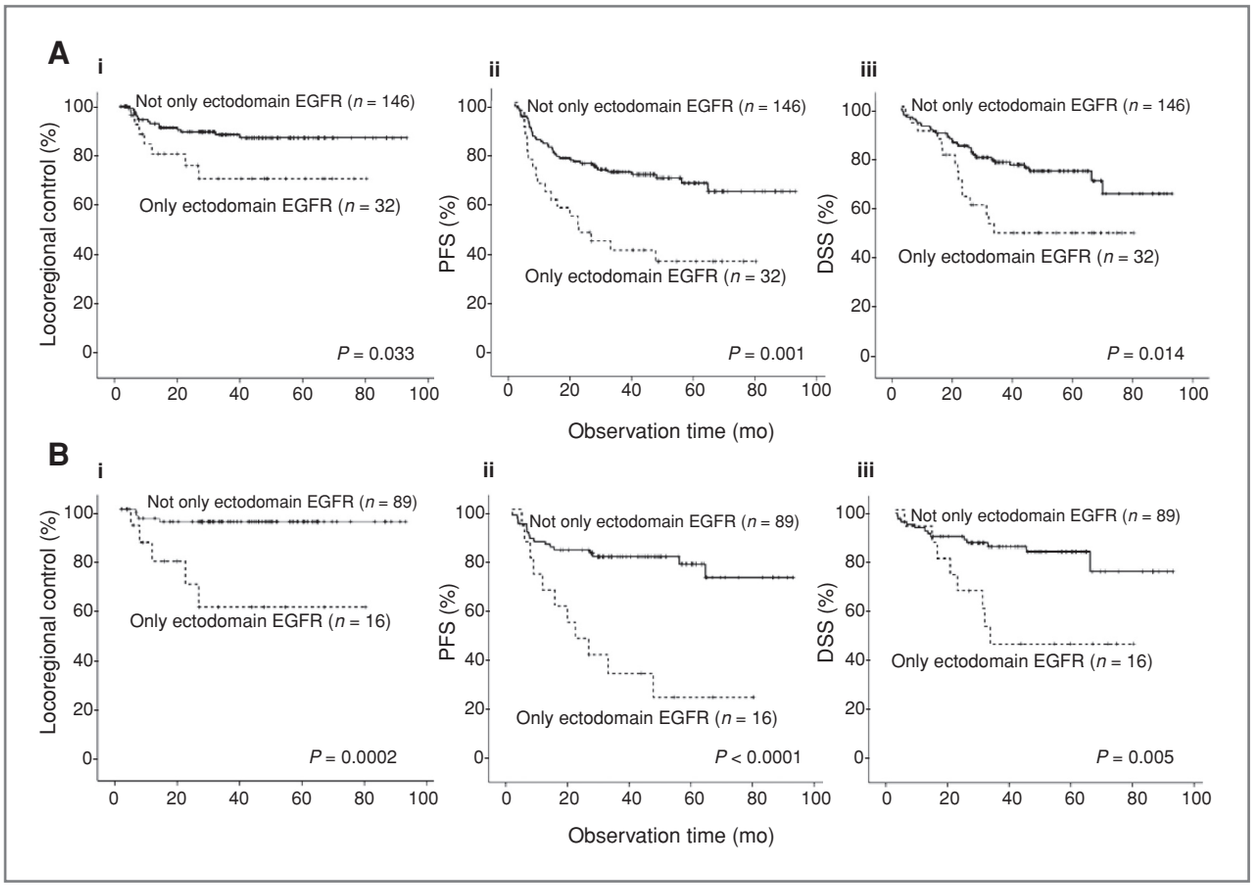

Figure 3. A, i–iii, Kaplan–Meier curves for locoregional control, PFS, and DSS, respectively, of cervical cancer patients with (dotted) and without (solid) membranous expression of only ectodomain isoforms of the EGFR. B, i–iii, Kaplan–Meier curves for locoregional control, PFS, and DSS, respectively of lymph node–negative cervical cancer patients with (dotted) and without (solid) membranous expression of only ectodomain isoforms of the EGFR. *P* values from the log-rank test and the number of patients are indicated.

The Linear Models for Microarray Data (LIMMA) software package (33) was used to find differentially expressed genes between 2 groups of patients on the basis of the Illumina data. Pathway analysis of these genes was done by allowing genes with $P < 0.01$ from the LIMMA analysis to generate a network of their known protein interactions, as listed in a set of 3 different protein interaction databases (34–36). A gene had a connection to a neighboring gene in the network if they had a protein interaction and if the neighboring gene had $P < 0.05$ from the LIMMA analysis. The Cytoscape software (37) was used to visualize networks with more than 3 members when including direct interactions of the neighboring genes, that is, first and second degrees of interaction.

Results

Expression and phosphorylation of EGFR isoforms

Totally, 135 tumors (76%) were EGFR positive by immunohistochemistry with the ECD-antibody, considering all isoforms (Table 1). Expression of the full-length receptor (ICD-antibody) was seen in 117 (66%) tumors (Table 1). The cytoplasmic and membranous staining was highly correlated for both antibodies ($P < 0.001$), and the results reported are therefore based on the membranous data only. A subgroup of 32 (18%) tumors showed membranous expression of only ectodomain EGFR isoforms without coexpressing the full-length receptor on the basis of positive ECD- and negative ICD-antibody membranous score (Table 1). Autophosphorylation of the full-length receptor occurred in almost half of the EGFR-positive cases (Table 1) and was associated with its expression level ($P = 0.003$). Nuclear expression of the EGFR protein was seldom seen, regardless of the antibody used, and none of the tumors displayed phosphorylated EGFR in the nucleus. Protein expression or autophosphorylation of the full-length receptor was not associated with tumor volume, stage, or lymph node status at the time of diagnosis. The expression of only ectodomain isoforms was neither related to stage nor lymph node status but was slightly higher in large than in small tumors ($P = 0.027$).

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Table 2. Cox regression analysis of membranous ectodomain EGFR and clinical variables

Factor	Univariate analysis[a]			Multivariate analysis[a,b]		
	P	HR	95% CI	*P*	HR	95% CI
All patients						
Locoregional control						
Ectodomain EGFR only	0.039	2.6	1.1–6.3	0.17	–	–
Tumor volume[c]	0.007	4.0	1.5–11.1	0.007	4.0	1.5–11.1
FIGO stage[d]	0.42	–	–	–	–	–
PFS						
Ectodomain EGFR only	0.001	2.5	1.4–4.2	0.049	1.8	1.0–3.4
Tumor volume[c]	0.0001	3.2	1.8–5.7	0.004	2.5	1.3–4.5
FIGO stage[d]	<0.0001	3.3	2.0–5.5	0.001	2.6	1.5–4.6
DSS						
Ectodomain EGFR only	0.016	2.1	1.2–3.9	0.066	–	–
Tumor volume[c]	0.0002	3.4	1.8–6.7	0.002	2.9	1.5—5.7
FIGO stage[d]	<0.0001	3.3	1.9–5.8	0.002	2.6	1.4—4.8
Lymph node–negative patients						
Locoregional control						
Ectodomain EGFR only	0.002	8.0	2.1–30.0	0.004	8.0	1.9–31.2
Tumor volume[c]	0.055	4.1	0.97–17.1	0.082	–	–
FIGO stage[d]	0.80	–	–	–	–	–
PFS						
Ectodomain EGFR only	0.0002	4.1	1.9–8.8	0.004	4.1	1.5–10.6
Tumor volume[c]	0.009	3.0	1.3–6.9	0.050	2.4	1.0–5.5
FIGO stage[d]	0.002	3.3	1.6–6.8	0.017	2.9	1.2–7.0
DSS						
Ectodomain EGFR only	0.008	3.2	1.4–7.8	0.141	–	–
Tumor volume[c]	0.027	3.0	1.1–7.9	0.027	3.0	1.1–7.9
FIGO stage[d]	0.01	3.0	1.3–7.0	0.156	–	–

Abbreviations: CI, confidence interval; FIGO, Federation International de Gynecologie et d'Obstetrique.

[a]*P* values and 95% CIs are listed.

[b]Only variables with a $P < 0.1$ in univariate analysis were included in the multivariate analysis.

[c]Tumor size was divided into 2 groups on the basis of the median volume of 43.2 (all patients) and 36.9 (lymph node–negative patients).

[d]FIGO stage was divided into 2 groups: 1b–2b and 3a–4a.

Three different protein products were detected by Western blotting, using the ECD-antibody to reveal all isoforms. A 60-kD product of the size of isoform C was seen in all tumors investigated, including 4 of those with only ectodomain isoforms in the membrane by immunohistochemistry. A ~90-kD product that could represent isoform D was detected as a weak band in 2 tumors, whereas a 170-kD product of the size of isoform A was detected in the tumors with positive ICD-antibody score by immunohistochemistry. The immunoblots were therefore in accordance with the immunohistochemistry findings, as shown by the pairwise data of four selected tumors in Fig. 2A and B for which P-89, P-97, and P-130 expressed only ectodomain isoforms whereas P-184 had additional expression of the 170-kD isoform.

In 10 of 13 tumors with only ectodomain isoforms by immunohistochemistry, the isoform C transcript was detected with RT-PCR (Fig. 2C, and data not shown). This transcript was also detected in 4 of 5 of the other tumors, for which 1 (P-184) is shown in Fig. 2C. The 60-kD ectodomain EGFR isoform detected with immunohistochemistry and Western blotting was therefore probably generated from alternative isoform C transcript and not by proteolytic cleavage of the full-length protein. Although hardly any other ectodomain isoforms were detected in the immunoblots, the isoform D transcript was present in almost all tumors and isoform B transcript in some (Fig. 2C). Moreover, all tumors had isoform A transcript, although no protein expression was seen in the tumors with only ectodomain isoforms.

Relationship to clinical outcome

At the time of evaluation, 61 patients (34%) had shown progressive disease, of whom 50 (28%) died because of the

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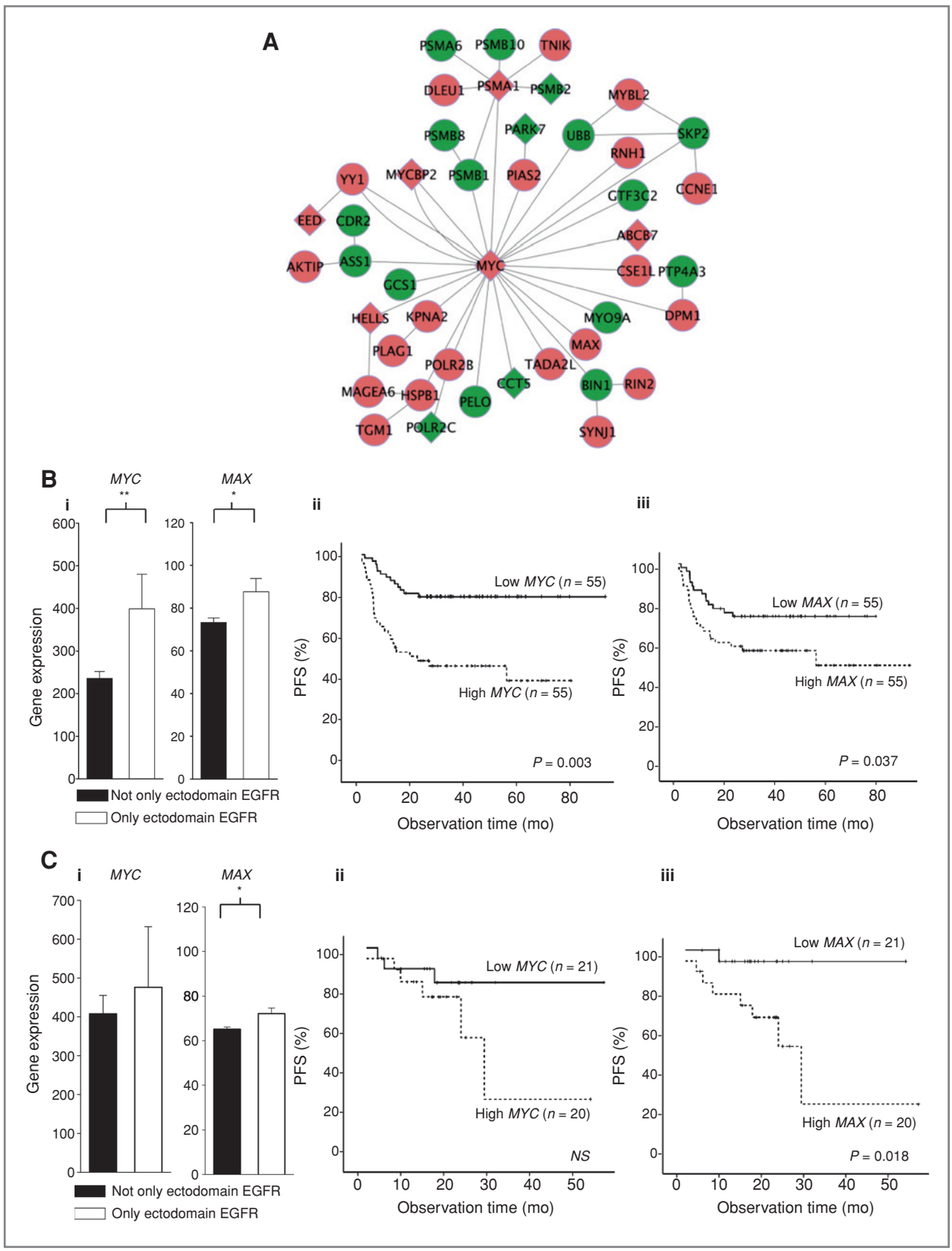

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actual cancer (Table 1). A total of 22 (12%) patients failed to achieve locoregional control, whereas 49 (28%) patients developed distant metastases. The immunohistochemistry data showed no correlation between the expression of the full-length receptor or its autophosphorylation status and PFS, locoregional control, or DSS, as shown for the autophosphorylation status in Supplementary Fig. S2. Neither was any correlation found for the total EGFR level. In contrast, patients with expression of only ectodomain isoforms had a significantly worse outcome than the others, regardless of endpoint (locoregional control, $P = 0.033$; PFS, $P = 0.001$; DSS, $P = 0.014$; Fig. 3A) and independent of clinical variables for PFS in multivariate analysis ($P = 0.049$; Table 2). These differences were significant only for the membranous and not the cytoplasmic expression of the ectodomain isoforms. Patients with EGFR-negative tumors had a clinical outcome similar to those positive for the full-length receptor (data not shown).

The association between membranous ectodomain isoforms and poor outcome was particularly strong for the lymph node–negative patients (Fig. 3B), whereas the relationship was lost for those with a diagnosis of lymph node metastases (Supplementary Fig. S3). In the lymph node–negative group, the locoregional control probability decreased from 95% to 62% ($P = 0.0002$), and the PFS and DSS probabilities decreased from 74% to 27% ($P < 0.0001$) and from 76% to 48% ($P = 0.005$), respectively, for patients expressing only ectodomain isoforms as compared with the others (Fig. 3B). Ectodomain isoform expression was an independent prognostic factor for PFS ($P = 0.004$) and had a much stronger impact than tumor volume ($P = 0.05$) and stage ($P = 0.017$) in this group. The expression of only ectodomain isoforms was not significant in multivariate analysis for locoregional control of all patients; however, it emerged as the only prognostic factor for locoregional control in lymph node–negative patients ($P = 0.004$; Table 2). Neither the total nor full-length EGFR level, or the autophosphorylation status, was significant in the lymph node–negative subgroup. Age and observation time were similar for the lymph node–negative and -positive groups (Supplementary Table S3)

Pathway analysis of tumors expressing only ectodomain EGFR isoforms

Signaling pathways associated with the membranous ectodomain isoforms were explored by comparing the gene expression profiles of tumors expressing only these isoforms ($n = 18$) with those of the others ($n = 92$) in 110 of the patients subjected to immunohistochemistry. Totally, 573 differentially expressed genes were identified at a significance level of $P < 0.01$. Networks were generated by selecting their known interaction partners among 2,655 genes that were differentially expressed at a significance level of $P < 0.05$. Four networks emerged, centered around the proto-oncogene *MYC*, the oncogene *CBL*, the growth factor receptor *FGFR3*, and the melanoma-antigen *MAGEA11*, respectively (Supplementary Fig. S4). These 4 genes were significantly upregulated in the tumors expressing only ectodomain isoforms, and their interaction partners in the networks were either significantly upregulated or downregulated, as visualized by red or green color in the figure. The result suggested differential signaling between the 2 groups of patients and activation of oncogenic pathways in the tumors with only ectodomain EGFR isoforms.

The MYC network had the highest number of differentially expressed interacting partners, including the *MYC*-associated factor *MAX*, which is required for *MYC* function (ref. 38; Fig. 4A and B). MYC can have both tumor-suppressive and oncogenic functions (38), however, downregulation of suppressors, such as the proapoptotic *BIN1* (Fig. 4A), suggests a tumorigenic role of *MYC* in the tumors with only ectodomain isoforms (38). Moreover, high expression of *MYC* and *MAX* was associated with poor PFS ($P = 0.003$, *MYC*; $P = 0.037$, *MAX*; Fig. 4B), supporting this hypothesis. For *MYC* the association to outcome was strongest in lymph node–negative patients ($P = 0.026$), but a clear tendency was seen also in lymph node–positive patients ($P = 0.084$; data not shown). For *MAX*, the association to outcome was present only in lymph node–negative cases ($P = 0.002$; data not shown), in accordance with the ectodomain EGFR data. Thus, the suggested aggressive phenotype of tumors expressing only ectodomain isoforms was confirmed by the gene expression data.

In the validation cohort, 6 of 29 (21%) tumors showed membranous expression of ectodomain EGFR without the coexpression of the full-length receptor. A tendency of elevated *MYC* expression and a significant increase in *MAX* expression ($P = 0.018$) were seen for these tumors [Fig. 4C (i)], consistent with the results in the basic cohort (Fig. 4A). Moreover, a clear difference in the survival curves based on *MYC* and *MAX* expression in 41 patients could be seen [Fig. 4C (ii and iii)], in accordance with the curves presented in Fig. 4B (ii and iii). The hypothesis of an aggressive phenotype of tumors expressing only

Figure 4. A, network of MYC interaction partners associated with the membranous expression of only ectodomain isoforms of the EGFR. Up- and downregulated genes in cervical cancer patients with only ectodomain isoforms are indicated with red and green color, respectively. Diamond, $P < 0.01$; circle, $P < 0.05$. B, i, gene expression of *MYC* and *MYC*-associated factor X (*MAX*) in cervical cancer patients with (white) and without (black) membranous expression of only ectodomain EGFR isoforms. Columns and bars represent mean and SE of 18 (white) and 92 (black) patients. **, $P < 0.01$; *, $P < 0.05$. B, ii and iii, Kaplan–Meier curves for PFS of cervical cancer patients with low (solid) or high (dotted) gene expression of *MYC* and *MAX*, respectively. C, i, gene expression of *MYC* and *MYC*-associated factor X (*MAX*) in cervical cancer patients in the validation cohort with (white) and without (black) membranous expression of only ectodomain EGFR isoforms. Columns and bars represent mean and SE of 6 (white) and 23 (black) patients, *, $P < 0.05$. C, ii and iii, Kaplan–Meier curves for PFS of 41 cervical cancer patients in the validation cohort with low (solid) or high (dotted) gene expression of *MYC* and *MAX*, respectively. *P* values from the log-rank test and the number of patients are indicated in the survival curves.

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ectodomain EGFR was thus confirmed in the independent group of patients.

Discussion

In a cervical cancer cohort of solely squamous cell carcinomas, membranous expression of ectodomain EGFR isoforms was found to be strongly associated with poor outcome after conventional chemoradiotherapy when not coexpressed with the full-length receptor. The 60-kD isoform C generated from alternative EGFR transcript was found to be the dominating ectodomain isoform, although isoform D appeared to be present in a few tumors. The suggested aggressive phenotype of tumors expressing only ectodomain isoforms in the membrane was confirmed by a large-scale gene expression analysis and validated in an independent cohort. By detection of EGFR-specific phosphorylation with PLA, we also showed that the autophosphorylation status had no influence on the clinical outcome. The present study is the first to compare the prognostic significance of EGFR isoforms and autophosphorylation status in the same cohort and to identify the expression of membranous ectodomain isoforms in the absence of the full-length receptor as a biomarker of aggressive disease. Moreover, our study indicates that a TK independent tumorigenic role of EGFR may be more important than TK activation in cancer patients. This finding may have implications for the use of adjuvant EGFR-targeted therapies.

The mechanisms underlying the strong association between the membranous ectodomain EGFR expression and aggressiveness are not clear. The ectodomain EGFR isoforms may lack a tumorigenic function *per se* and rather reflect the activation of oncogene(s) other than EGFR because membranous expression of the full-length receptor is absent. Such oncogenes could be *MYC*, *CBL*, *FGFR3*, and/or *MAGEA11*, as these genes were significantly upregulated and generated interaction networks in the tumors with only ectodomain EGFR. Several studies have indicated an important role of *MYC* in the progression of cervical cancers (39, 40), consistent with the correlation between *MYC* expression and clinical outcome in our work. Moreover, *CBL* is a key player in numerous oncogenic signaling pathways (41), *FGFR3* is known to promote tumor progression (42), and *MAGEA11* might be involved in the regulation of hypoxia inducible factors and thereby promote tumor angiogenesis and glycolysis (43). The activation of these proteins in the tumors with ectodomain EGFR isoforms is therefore in accordance with an aggressive phenotype. It should also be noted that the network analysis favors pathways for which a large number of interaction partners is known. It is therefore possible that pathways not identified here are more important for the aggressive ectodomain EGFR phenotype.

An oncogenic function of the membranous ectodomain EGFR isoforms in the absence of the full-length receptor is also possible. Although only isoform D has been reported to be cell membrane associated (21, 44), it is tempting to speculate that both isoforms C and D may interact with and stabilize membranous oncoproteins and thereby promote aggressiveness, as was recently shown for the interaction between the ECD of the full-length EGFR and the glucose transporter SGLT1 (24). *MYC* is known to directly enhance the expression of glucose and glutamate transporters (38), which might be interaction partners for ectodomain EGFR. The regulation of energy metabolism could therefore be a possible arena of cooperation between these EGFR isoforms and *MYC*. Moreover, the activation of *CBL*, *FGFR3*, and *MAGEA11* in the tumors with only ectodomain EGFR isoforms points to other pathways where the isoforms may participate through unknown mechanisms. It is also possible that the expression of ectodomain isoforms and activation of these pathways are independent events that lead to increased aggressiveness when present in the same tumors.

It seemed to be crucial for the aggressive tumor phenotype whether or not the ectodomain EGFR isoforms were coexpressed with the full-length receptor. The full-length EGFR was expressed at the transcriptional level but not as membranous protein in the tumors with only ectodomain isoforms in the membrane, indicating that the receptor is regulated at the protein level. *CBL* was upregulated in tumors with only ectodomain EGFR and plays an important role in downregulation of the full-length EGFR by ubiquitylation (41). The increased expression of *CBL* could thus possibly explain the lack of full-length EGFR protein in these tumors. It is further possible that the ectodomain isoforms dimerize with the full-length receptor once they are coexpressed, possibly retaining the ectodomain isoforms from their potential oncogenic role.

Membranous ectodomain EGFR isoforms were associated with aggressiveness only in tumors that had not yet detectable lymph node metastases. Previous studies have indicated differences in the biology between lymph node–negative and -positive cervical tumors (30). It is therefore plausible that the prognostic value of individual tumor markers may differ between these groups, as has been shown for early- and late-stage cervical cancer (39). Thus, ectodomain EGFR may be a marker of and possibly be involved in crucial steps in tumor progression toward metastatic disease, whereas other molecular processes in the tumor may become more important for its aggressiveness when spreading has occurred. In line with this hypothesis, preliminary studies in our laboratory suggest that the membranous expression of ectodomain EGFR isoforms alone is associated with poor clinical outcome also in lymph node–negative operable cervical tumors with an invasion depth above 1 cm (data not shown). Few characteristics of aggressive lymph node–negative cervical tumors have been identified, for which hypoxia seems to be among the strongest prognostic factors (45). It might therefore be possible that membranous ectodomain EGFR is involved in retaining a hypoxic phenotype in these tumors.

Depending on the binding site of the antibodies used for immunohistochemistry, different isoforms were detected

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in our study. This shows that the antibody binding site greatly influences the results when the prognostic impact of EGFR is investigated, which may explain some of the apparent inconsistency in previous work (1, 2, 6–10). Moreover, the expression of ectodomain isoforms in the membrane may contribute to the poor concordance between EGFR expression measured by ECD antibodies and responsiveness to TK inhibitors, as remarked by Wilken and colleagues (46).

The lack of correlation between the EGFR autophosphorylation status and outcome indicates that the TK activation plays a minor role compared with the ectodomain isoforms in disease progression. In contrast to our findings, Noordhuis and colleagues (9) reported a correlation between the autophosphorylation status and locoregional control in cervical cancer. This apparent inconsistency may be explained because we used a highly EGFR-specific method for assessing the autophosphorylation status. Hence, phospho-antibodies against TK sites are often hampered by unspecific binding due to a large site similarity across family members (47, 48). Another explanation could be that we included a more homogeneous patient group with respect to stage, histology, and therapy, because it has been shown that such factors may influence the prognostic significance of EGFR (39, 49). It should be mentioned, however, that although Tyr1068 phosphorylation probably is a good indicator of TK activation, the phosphorylation of other sites such as the SRC-dependent Tyr845 (50) might have prognostic value and should be explored in cervical cancers. The balance and differential heterodimerization of the various ERBB receptors may also be important (10); thus, it is possible that the phosphorylated EGFR promotes aggressiveness only in cases of a specific dimerization partner.

The strong prognostic impact of membranous ectodomain EGFR isoforms in lymph node–negative patients was independent of existing clinical markers. The lymph node–negative patients typically constitute the major subgroup of cervical cancer patients subjected to curative chemoradiotherapy. There are currently few means of identifying patients in this subgroup with a high risk of relapse, especially with respect to locoregional control for which tumor stage and volume are of minor value. Our study suggests that the ectodomain EGFR could be a well-needed biomarker of the resistance to conventional chemoradiotherapy for these patients. In addition to being the only significant parameter for locoregional control, the ectodomain EGFR had a much stronger prognostic impact than tumor stage and volume for progressive disease. This biomarker would therefore be useful for selection of patients for more aggressive radiotherapy and/or adjuvant targeted therapy. It is also suggested from our findings that, in an effort to improve the clinical outcome by adjuvant EGFR-targeted therapy, TK inhibitors may not have the desired therapeutic effect in lymph node–negative cervical cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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