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Correlation between human epidermal growth factor receptor family (EGFR, HER2, HER3, HER4), phosphorylated Akt (P-Akt), and clinical outcomes after radiation therapy in carcinoma of the cervix ☆

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Abstract

Objective. To investigate prognostic significance of and correlations between HER1 (EGFR), HER2 (c-erb-B2), HER3 (c-erb-B3), HER4 (c-erb-B4), and phosphorylated Akt (P-Akt) in patients treated with radiation for cervical carcinoma.

Methods. Fifty-five patients with stages I–IVA cervical carcinoma were treated with definitive radiotherapy. Tumor expression of each biomarker was quantitatively scored by an automated immunohistochemical imaging system. Parametric correlations were performed between biomarkers. Univariate and multivariate analysis was performed with disease-free survival (DFS) and overall survival (OS) as primary endpoints.

Results. Correlations were observed between expression of HER2 and HER4 ($P = 0.003$), and HER3 and HER4 ($P = 0.004$). Decreased HER2, HER4, and P-Akt expressions were significant for diminished DFS on univariate analysis ($P = 0.04$, $P = 0.008$, and $P = 0.02$, respectively). Increased EGFR, and diminished HER2, HER4, and P-Akt expression were significant or showed trends toward significance for diminished OS on univariate analysis ($P = 0.07$, $P = 0.008$, $P = 0.09$, and $P = 0.08$, respectively). After controlling for pretreatment factors, multivariate analysis revealed HER2 associated with improved OS ($P = 0.05$).

Conclusions. These data emphasize that significant correlations exist between the differential expression of various HER family receptors. Multivariate analysis revealed only increased HER2 expression associated with improved OS after controlling for pretreatment clinical factors. These data emphasize the importance of continued basic and translational research on the HER family of receptors in cervical carcinoma.

Keywords: EGFR; HER2; HER3; HER4; Cervix cancer

Introduction

The human epidermal growth factor receptor (HER) family plays a key role in regulation of mammalian cell survival, proliferation, adhesion, and differentiation [1–4]. This HER family of receptor tyrosine kinases comprises four structurally related transmembrane receptors: HER1 (EGFR or erbB1), HER2 (HER2neu or c-erb-B2), HER3 (c-erb-B3), and HER4 (c-erb-B4). All members of the family have an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain which for HER3 is nonfunctioning [1,5]. In response to ligand specific binding, these receptors act by forming hetero- or homodimers which thereby initiate tyrosine kinase activity in the intracellular domain. This amplification of tyrosine kinase activity promotes the phosphorylation of several tyrosine residues in the C-terminus which leads to a complex signaling cascade. This signaling cascade has been

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shown to result in cell proliferation, differentiation, migration, adhesion, protection from apoptosis, and transformation [1,5,6].

The oncogenic pathway of some cells is thought to be initiated as a result of HER family receptor mutation, overexpression, structural rearrangements, and/or relief of normal regulatory or inhibitory pathways [1,4–7]. The presence of EGFR and Her2neu receptors has also been associated with accelerated tumor progression and resistance to therapy for multiple types of malignancies [1,5,6] and small molecule inhibitors of their activity are being examined as targeted therapy in cancer patients. In addition, overexpression of all four receptors has been observed in malignancies such as breast cancer [4,5]. Of note, HER4 has in multiple reports been linked to a good prognosis and a longer disease-free interval [3,4,8]. The causal relationship of this receptor network to disease progression and resistance to therapy provides a rationale for targeting this signaling system with tumor-selective strategies.

The phosphatidylinositol 3-kinase (PI3K) family of enzymes has also been well studied with respect to promotion of cellular growth and survival in cancer cells [6,9,10]. These kinases can be activated from cell surface growth factor receptors (such as the HER family receptors) and are known to play important roles in the balance between cell survival and apoptosis. One known fundamental cellular response of the activation of PI3K in vitro and in vivo is the downstream phosphorylation of Akt [10]. Increased intratumoral phosphorylated Akt (P-Akt) has been linked to decreased radiation responsiveness in various malignancies, including head and neck squamous cell carcinoma, lung carcinoma, glioblastoma, and prostate and breast cancer [6,9,10]. Therefore, it has also been postulated that the inhibition of PI3K may provide an additional targeted approach to therapy for this disease process.

We have recently reported that increased expression of HER2 and decreased expression of EGFR membranous staining correlate with improved overall survival in patients with carcinoma of the cervix. We also found that overexpression of HER2 was correlated with adenocarcinoma, and overexpression of EGFR correlated significantly with squamous cell histology [11]. Few studies have evaluated the prognostic utility of multiple HER family biomarkers in carcinoma of the cervix for patients treated in a homogeneous fashion (such as definitive radiotherapy alone). The goals of this study were to evaluate the correlation between members of the HER family and P-Akt in carcinoma of the cervix, and to analyze their prognostic significance in regard to disease-free survival and overall survival by both univariate and multivariate analysis.

Materials and methods

Fifty-five patients who received radiotherapy alone with definitive intent from 1981–1996 on whom tissue blocks were available were included within this study. The patients included within this group were comprised of FIGO stage IB through IVA carcinoma of the cervix. All patients were treated at a single radiation therapy center with standard external beam radiation techniques (with MV photon energies) and the brachytherapy component was delivered with low-dose rate brachytherapy for all patients included in the analysis. The specifics of radiotherapy treatment technique and dose for this patient cohort are described elsewhere [11]. Approval for this study was obtained from the LDS Hospital Institutional Review Board (IRB). Patients who received concurrent chemotherapy or prior surgery for curative intent were excluded from the analysis to provide a more homogenous population for analysis. Medical charts and the hospital registry were also reviewed for clinical parameters and disease status.

The presence of tumor and histopathologic grade was verified by one pathologist prior to performing the specific immunohistochemical analyses. In order to evaluate significant correlations between tumor histology and biomarker expression, patients were divided into squamous cell carcinoma and adenocarcinoma groups (including adenosquamous carcinoma). Immunohistochemistry was performed for EGFR, HER2, HER3, HER4, and P-Akt. The cellular expression of each biomarker was then quantitatively scored by ChromaVision cellular imaging technology for each patient (intensity scale based on pixel density, 0–16000 for EGFR, 0–4.0 for HER2, 0–4.0 for HER3, 0–4.0 for HER4, and 0–16,000 for P-Akt). Overexpression of EGFR, HER2, HER3, HER4, and P-Akt was defined as $\geq$7380, $\geq$2.0, $\geq$0.8, $\geq$2.4, and $\geq$3780 (intensity scale), respectively.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 5-μm thick sections were prepared from biopsy specimens. Immunohistochemical stains were individually assessed without knowledge of patient outcome. Each slide was first deparaffinized and heated in citrate buffer. After cooling and rinsing in a Tris buffer, immunohistochemical staining was performed with an automated immunohistochemical autostainer.

Detection of EGFR was performed with a secondary mouse anti-immunoglobulin linked to biotin following incubation with streptavidin linked to horseradish peroxidase. The EGFR detection kit was obtained from Dako (M3563) and was used in a concentration of 1:200. Detection of HER2 was performed with a secondary rabbit anti-immunoglobulin linked to biotin following incubation with streptavidin linked to horseradish peroxidase. The HER2 detection kit was obtained from Dako (A0485) and was used in a concentration of 1:200. Detection of HER3 and HER4 was performed with secondary mouse anti-immunoglobulins linked to biotin following incubation with streptavidin linked to horseradish peroxidase. The HER3 and HER4 detection kits were obtained from NeoMarkers (MS-725-P and MS-637-P, respectively) and were used in a concentration of 1:20 and 1:40, respectively. Detection for P-Akt was performed with a secondary rabbit anti-immunoglobulin linked to biotin following incubation with streptavidin linked to horseradish peroxidase. The P-Akt detection kit was obtained from Cell Signaling (9277) and was used in a concentration of 1:50. Color development for all assays was accomplished with diaminobenzidine as the chromagen. Due to the limited quantity of biopsy specimen in select cases, specific immunohistochemically stained slides were unable to be analyzed either because of lack of specimen, an abundance of necrotic tissue, or too little carcinoma to be accurately stained and quantitatively scored by Chromavision software.

Statistics

Parametric (Pearson's correlation) and nonparametric correlations (Spearman's rho) were performed between biomarkers. Univariate and multivariate Cox proportional hazards modeling were performed with disease-free survival (DFS) and overall survival (OS) as the primary endpoints, and each biomarker was evaluated for correlation between various pretreatment clinical factors. The Kaplan–Meier method was utilized to evaluate for differences in OS and DFS for specific prognostic factors. The median time of follow-up was 24 months for all patients and 69 months for living patients. Differences were considered significant when the probability of error was below 5% ($P < 0.05$).

Results

Table 1 lists the pretreatment characteristics of this series of 55 patients with FIGO stage IB through IVA carcinoma of the

Table 1
Patient and radiation treatment characteristics (N = 55)

Characteristics	N	Median	SEM[a]	Range
FIGO stage				
IB	12			
II	22			
III	17			
IVA	4			
Histology				
Squamous	48			
Adenocarcinoma	5			
Adenosquamous	2			
Grade				
1	2			
2	34			
3	19			
Follow-up interval (months)				
All patients		24	7	1–227
Alive patients		69	15	7–227
Total dose to point A (cGy)		8038	250	800–12000
Total treatment time (days)		44	1.6	6–77

[a] SEM indicates standard error of the mean.

cervix. All patients were treated with curative intent with definitive radiotherapy alone. Within this population, 48 patients had a pathologic diagnosis of squamous cell carcinoma, 5 adenocarcinoma, and 2 adenosquamous carcinoma. The median dose to point A was 8038 cGy.

Parametric and nonparametric correlations were performed between biomarkers (EGFR, HER2, HER3, HER4, and P-Akt) and between pretreatment tumor characteristics (Table 2). Increased expression of EGFR correlated significantly with squamous cell carcinoma histology (P = 0.05), and increased expression of HER2 correlated significantly with adenocarcinoma histology (P = 0.02). We also found that increased HER2 expression had statistically significant correlations with increased expression of HER4 and was inversely correlated with pretreatment stage of disease (P = 0.003 and P = 0.004, respectively). Direct correlations between expression of HER3 and HER4, and HER4 and P-Akt were observed (P = 0.004 and P = 0.07, respectively). We did not find significant correlations between expression of P-Akt and the EGFR, HER2, and HER3 biomarkers.

Mean biomarker expressions of HER3, HER4, and P-Akt were compared for squamous cell carcinoma and adenocarcinoma groups (Fig. 1). Increased mean expression of HER3 correlated significantly with squamous cell carcinoma (P = 0.02). There was not a statistically significant difference detected in mean HER4 or P-Akt expression between the squamous cell carcinoma and adenocarcinoma histology groups (P = 0.31 and P = 0.15, respectively).

The Kaplan–Meier method revealed statistically significant differences in DFS and OS for specific biomarkers (Figs. 2 and 3). Increased expression of HER4 and P-Akt were significant for improved DFS (Fig. 2) on univariate analysis (P = 0.008 and P = 0.015, respectively). Increased expression of HER4, and increased expression of P-Akt also showed trends toward statistically significant differences in OS (Fig. 3) on univariate analysis (P = 0.09 and P = 0.08, respectively).

Multivariate analyses (Table 3) were performed with overall survival (OS) and disease-free survival (DFS) as the primary endpoints. After controlling for pretreatment factors (stage,

Table 2
Correlations between Akt, EGFR family, and pretreatment variables

		Akt	EGFR	HER2	HER3	HER4	Histology	Stage	Grade
Akt	Correlation	1	0.103	0.218	−0.071	0.252	0.195	−0.258	−0.075
	P	.	0.487	0.109	0.609	0.069	0.154	0.058	0.587
	N	55	48	55	54	53	55	55	55
EGFR	Correlation	0.103	1	−0.070	−0.046	−0.215	−0.285	−0.036	−0.063
	P	0.487	.	0.639	0.761	0.152	**0.050**	0.809	0.669
	N	48	48	48	47	46	48	48	48
HER2	Correlation	0.218	−0.070	1	0.094	0.395	0.303	−0.385	−0.058
	Sig. (2-tailed)	0.109	0.639	.	0.500	**0.003**	**0.024**	**0.004**	0.671
	N	55	48	55	54	53	55	55	55
HER3	Correlation	−0.071	−0.046	0.094	1	0.392	−0.176	−0.016	−0.005
	P	0.609	0.761	0.500	.	**0.004**	0.204	0.908	0.970
	N	54	47	54	54	52	54	54	54
HER4	Correlation	0.252	−0.215	0.395	0.392	1	0.133	−0.223	0.075
	P	0.069	0.152	**0.003**	**0.004**	.	0.343	0.109	0.595
	N	53	46	53	52	53	53	53	53
Histology	Correlation	0.195	−0.285	0.303	−0.176	0.133	1	−0.103	−0.119
	P	0.154	**0.050**	**0.024**	0.204	0.343	.	0.453	0.388
	N	55	48	55	54	53	55	55	55
Stage	Correlation	− 0258	−0.036	−0.385	−0.016	−0.223	−0.103	1	−0.117
	P	0.058	0.809	**0.004**	0.908	0.109	0.453	.	0.393
	N	55	48	55	54	53	55	55	55
Grade	Correlation	−0.075	−0.063	−0.058	−0.005	0.075	−0.119	−0.117	1
	P	0.587	0.669	0.671	0.970	0.595	0.388	0.393	.
	N	55	48	55	54	53	55	55	55

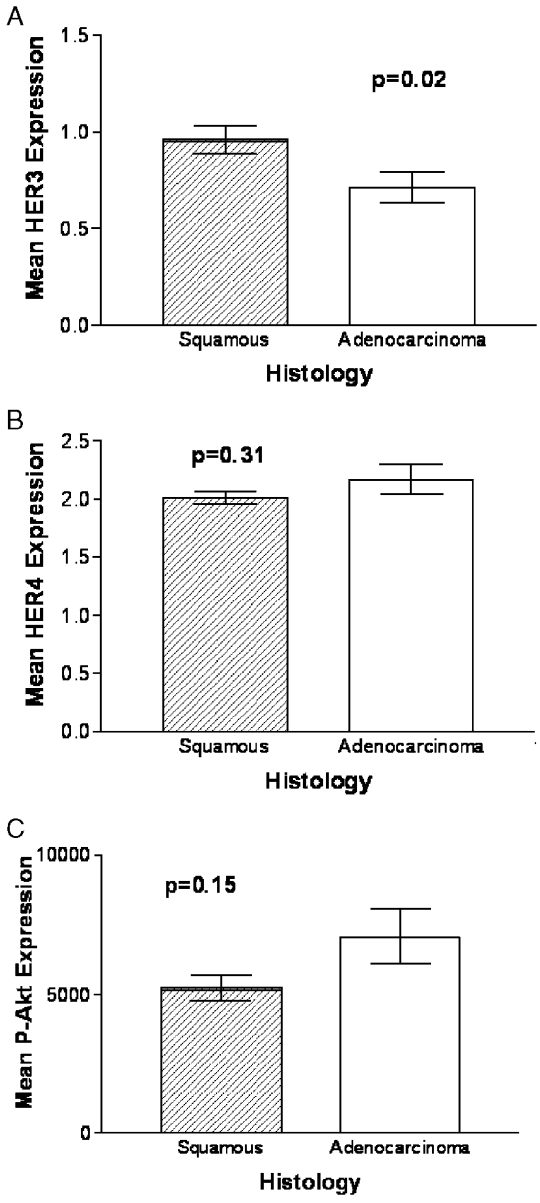

Fig. 1. Comparison of biomarker expression by histologic subtypes of cervical carcinoma (bars illustrate mean biomarker expression ± SEM). (A) HER3 expression, (B) HER4 expression, (C) P-Akt expression.

grade, and histology) in our model, multivariate analysis of all biomarkers revealed that only increased expression of HER2 was associated with improved OS (HR = 0.31, 95% CI = 0.10–0.99, $P = 0.05$). There were no recorded "events" of recurrence of disease for those patients with increased expression of HER4. Due to this observation, the Cox regression model was unable to calculate a hazard ratio and confidence interval as increased HER4 expression perfectly predicted disease-free survival for that patient cohort.

Discussion

The amplification, overexpression, and coexpression of the HER family receptors have been implicated in the genesis or progression of human mammary, ovarian, gastric cancers and glioblastoma [12–15]. In the past, mixed results have been published as to the prognostic value of their expression. For example, EGFR and HER2 have both been shown in some studies to be independent predictors of a poor prognosis in carcinoma of the cervix [16–23]. Although, other studies have also revealed no statistically significant correlation with an adverse outcome [16,24–27]. Mixed results have also been published on the prognostic significance of these markers in other gynecologic sites [2,28–32]. Although extensive cellular crosstalk between individual HER family members exists, recent coexpression studies have begun to elucidate the cellular

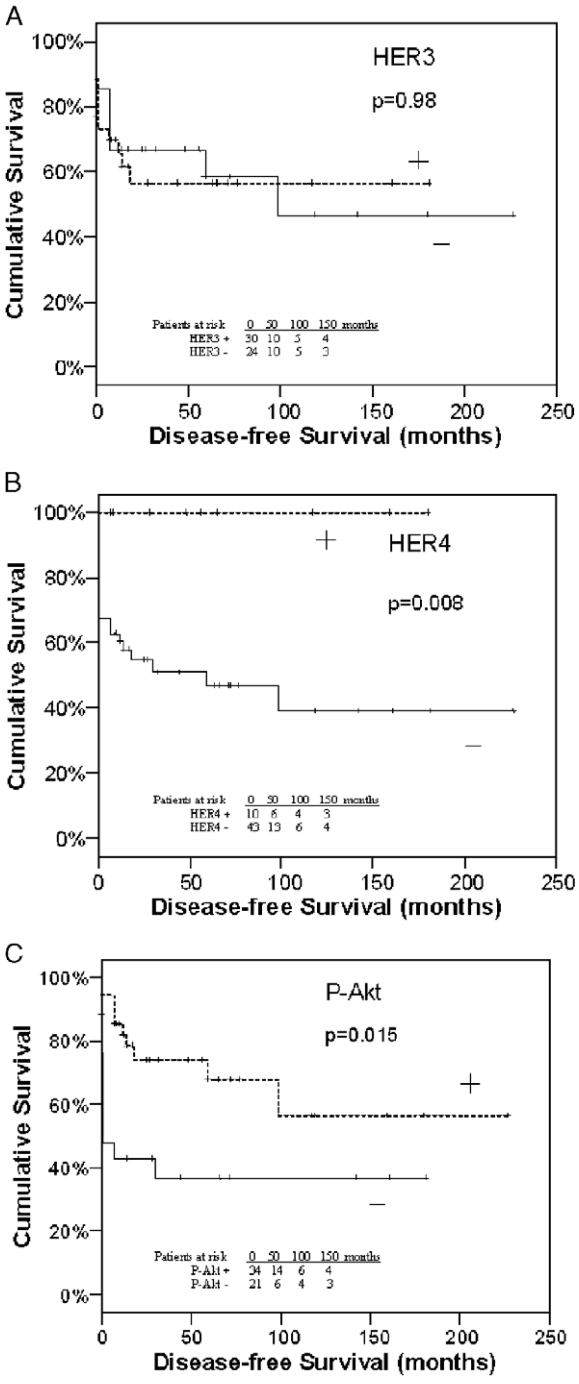

Fig. 2. Kaplan–Meier curves for disease-free survival related to biomarker expression in carcinoma of the cervix. (A) HER3 expression, (B) HER4 expression, (C) P-Akt expression.

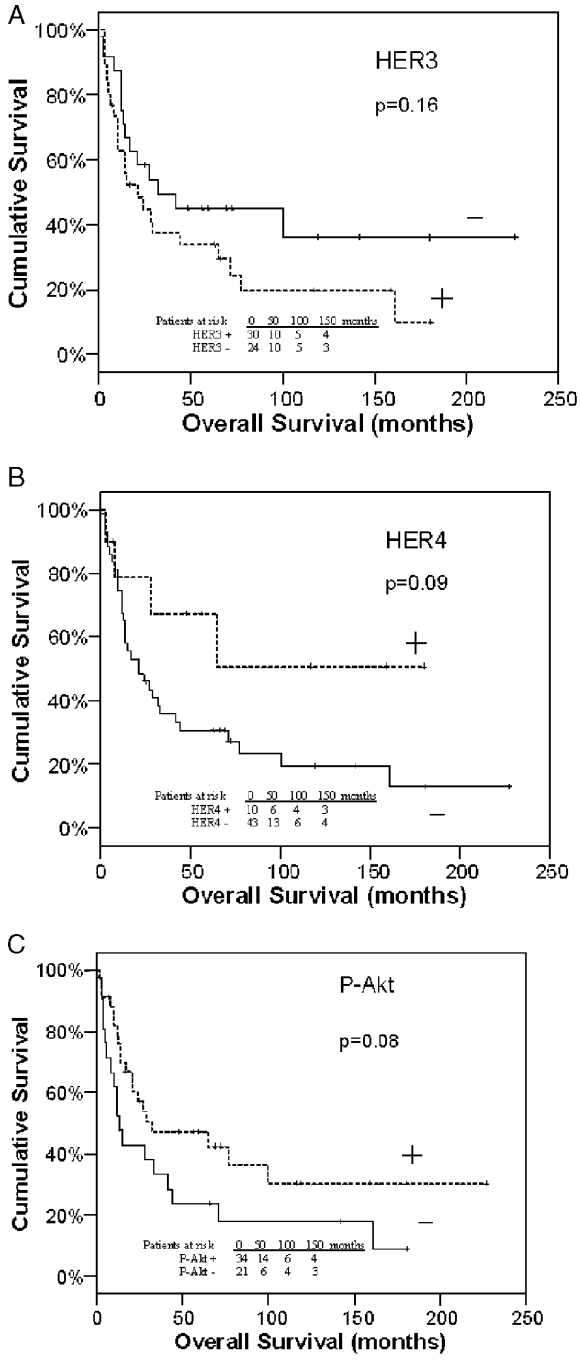

Fig. 3. Kaplan–Meier curves for overall survival related to biomarker expression in carcinoma of the cervix. (A) HER3 expression, (B) HER4 expression, (C) P-Akt expression.

signaling capacities of individual homodimeric and heterodimeric complexes [33,34].

In our analysis, correlations were performed between biomarkers (EGFR, HER2, HER3, HER4, and P-Akt) and between pretreatment tumor characteristics. We did find that increased HER2 expression had statistically significant correlations with increased expression of HER4 ($P = 0.003$), and that direct correlations between expression of HER3 and HER4, and HER4 and P-Akt were observed ($P = 0.004$ and $P = 0.07$, respectively). While all four HER receptors are topologically similar, other investigators have noted specific receptor characteristics that are significantly different between each subtype [1,3,34–36]. EGFR is known to preferentially form a heterodimer with HER2 to synergistically increase signal transduction. Although HER2 is also the preferred heterodimeric partner for the other family members, there is no known specific HER2 ligand. The extracellular domain of HER2 has been described to be in a dimer-ready state without the addition of bound ligand, and thus HER2's active tyrosine kinase on the intracellular domain is at the disposal of the other HER family members after they bind ligand. This unique situation allows the other HER receptors to elegantly modulate HER2 signaling potential and in this way HER2 becomes a common signal-amplifying cofactor in the HER family model [1,3,4,35,36]. HER3 is unique in that it does not exhibit an intrinsic kinase activity, and depends upon a heterodimeric partnership with other HER family members to provide tyrosine kinase activity. The partnering receptor's tyrosine kinase phosphorylates the signal-producing tyrosines within the HER3 C-terminus, which in turn leads to signal transduction. The preferred partner for HER3 (ligand bound) is known to be HER2, but heterodimers with EGFR and HER4 have been described [3,4].

Recently developed molecularly targeted cancer therapies are based on the known functions of the HER family receptors [1,5,9]. The first strategy involves development of humanized monoclonal antibodies against the receptor's extracellular domain, which in turn downregulates the receptors and blocks further ligand binding. The second approach is to generate adenosine triphosphate-mimetics that compete with adenosine triphosphate for binding to the receptor's kinase pocket and disable the ability of the dimer complex to transduce intracellular signals [1,5]. Early clinical studies for multiple tumor sites have shown that both approaches alone or in combination with standard anticancer therapies have altered the natural history of EGFR-expressing cancers with little toxicity to the tumor-bearing host [1]. Examples of monoclonal antibodies in clinical use include trastuzumab [37] (an anti-

Table 3
Multivariate analysis of biomarkers with disease-free survival and overall survival endpoints

Variable	Hazard ratio	95% CI	*P* value
Disease-free survival			
EGFR	1.05	0.38–2.89	0.92
HER2	0.35	0.08–1.65	0.19
HER3	0.72	0.30–1.73	0.46
HER4	<0.00001		0.99
P-Akt	0.54	0.22–1.33	0.18
Overall survival			
EGFR	1.37	0.63–2.96	0.42
HER2	0.32	0.10–0.98	*0.05
HER3	1.57	0.76–3.22	0.22
HER4	0.71	0.22–2.25	0.56
P-Akt	0.61	0.31–1.21	0.16

Multivariate Cox regression models are controlled for tumor histology, stage, and grade.

* Correlation is significant at the 0.05 level.

HER2 antibody) and cetuximab (activity in colorectal cancer) [38]. In both instances, as predicted by preclinical data, there have been additive to super-additive effects with chemotherapy [38]. Tyrosine kinase inhibitors, such as ZD1839 (IressaTM) and erlotinib (OSI-774), have also demonstrated activity in early preclinical and clinical studies [39,40]. Further work in the area of gynecologic oncology is needed to elucidate the utility of these agents in carcinoma of the cervix. A key future challenge in determining guidelines for use of such targeted therapies will be in the elucidation of mechanisms for appropriate patient selection. In this age of molecularly targeted therapy, our understanding of the complex interplay between signaling systems (such as the HER family receptors) will be an important determinant of therapeutic outcomes.

Although conflicting results are evident in the literature, it is noteworthy that in our analysis HER2 overexpression was a favorable prognostic factor on both univariate and multivariate analysis for the OS endpoint. After controlling for pretreatment factors (stage, grade, and histology) in our model, multivariate analysis of all biomarkers revealed that only increased expression of HER2 was associated with improved OS (HR = 0.31, 95% CI = 0.10–0.99, $P = 0.05$). Leung et al. found in their analysis that HER2 played a more significant role in tumor progression and invasion for carcinoma of the cervix than EGFR, but neither HER2 nor EGFR was found to carry any prognostic significance [24]. The absence of correlation between clinical prognosis and HER2 expression was also described by Ngan et al. [25]. In other previous studies, elevated HER2neu has correlated with tumor size, local failure, and diminished survival in cervical cancer [41]. Kihana et al. found in their analysis of 44 patients with cervical adenocarcinoma that increased expression of HER2 correlated with a poorer prognosis [21]. It must also be mentioned that only a quantitative analysis of receptor expression was performed in this study, although it is understood that the presence or absence of specific ligand also plays an important role in cellular response. In the majority of these previous clinical studies, tumors with adenocarcinoma were evaluated. In our study population, the vast majority of tumors consisted of squamous cell histology and may account for the differences in biomarker correlates.

In general, the goals of this study have been reached although it remains limited in its statistical power. We understood from the outset of this study that the outcome would primarily serve to be a hypothesis generating future testing of specific biomarkers or biomarker combinations on a larger scale. The reported differences in disease-free survival and overall survival are also a function of the low power of this study. Assuming a sample size of 27 in each group and with a total number of events required equal to 30, a 0.05 level log-rank test for equality of survival curves will have 20% power to detect the difference between both groups (assuming a constant hazard ratio of 1.515). In order to further study these issues, we are interested in performing further immunohistochemical analyses with specimen from prospectively acquired clinical databases and tissue repository. We also encourage further studies by other research groups of these promising biomarkers.

In conclusion, we found that increased expression of HER2 identified patients with an improved OS on both univariate and multivariate analysis after controlling for pretreatment clinical factors. Significant correlations also exist in cervical cancer specimens between expressions of transmembrane receptors in the EGFR family. These data emphasize that each member of the EGFR family exhibits differential expression in tumor tissue. Due to conflicting results in the literature, further coexpression studies will be needed to elucidate the cellular signaling capacities of individual homodimeric and heterodimeric complexes. In this age of molecularly targeted therapy, our understanding of the complex interplay between signaling systems (such as the HER family receptors) will be an important determinant of therapeutic outcomes. These data emphasize the importance of continued basic and translational research on the EGFR family of receptors in carcinoma of the cervix.

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