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Expression of HER2neu (c-erbB-2) and epidermal growth factor receptor in cervical cancer: prognostic correlation with clinical characteristics, and comparison of manual and automated imaging analysis ☆

Christopher M. Lee,[a] R. Jeffrey Lee,[b] Elizabeth Hammond,[c] Alexander Tsodikov,[d] Mark Dodson,[e] Karen Zempolich,[e] and David K. Gaffney[a,*]

[a] *Department of Radiation Oncology, University of Utah, Salt Lake City, UT 84132, USA*
[b] *Department of Radiation Oncology, LDS Hospital, Salt Lake City, UT, USA*
[c] *Department of Pathology, LDS Hospital, Salt Lake City, UT, USA*
[d] *Department of Biostatistics, Huntsman Cancer Institute, Salt Lake City, UT, USA*
[e] *Department of Gynecologic Oncology, University of Utah, Salt Lake City, UT, USA*

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Abstract

Objectives. To evaluate HER2neu and epidermal growth factor receptor (EGFR) expression with respect to overall survival and disease-free survival (DFS), and correlate expression with pretreatment factors. Comparative evaluations of manual and automated immunohistochemical imaging systems for HER2neu and EGFR expression were made.

Methods. Fifty-five patients with stages I–IVA carcinoma of the cervix were treated with definitive radiation therapy. Immunohistochemistry was performed for HER2neu and EGFR, and scored by both manual and automated methods. Univariate and multivariate analyses were performed with disease-free survival (DFS) and overall survival (OS) as primary endpoints, and biomarkers were evaluated for correlation between prognostic factors.

Results. Strong correlations in HER2neu and EGFR protein expression were observed between digitally and manually analyzed staining ($P \leq 0.0001$). Increased FIGO stage and decreased HER2neu expression were significant for reduced DFS on univariate analysis ($P \leq 0.001$ and $P = 0.03$, respectively). Increased FIGO stage, decreased HER2neu expression, and increased membranous staining of EGFR were significant for diminished OS on univariate analysis ($P \leq 0.0001$, $P = 0.002$, and $P = 0.043$, respectively). Multivariate analysis revealed only increased membranous staining of EGFR associated with diminished DFS and OS ($P = 0.046$ and $P = 0.012$, respectively). Overexpression of HER2neu correlated significantly with adenocarcinoma, and overexpression of EGFR correlated significantly with squamous cell carcinoma histology ($P = 0.038$ and $P = 0.035$). Inverse correlations were observed between HER2neu expression and clinical stage, EGFR membranous staining, and EGFR distribution ($P = 0.007$, $P = 0.006$, and $P = 0.034$, respectively).

Conclusions. Increased expression of HER2neu and decreased EGFR membranous staining identified patients with improved DFS and OS on univariate analysis, although only decreased EGFR membranous staining was significant on multivariate analysis. We also found strong correlation of results between manually and automated imaging methods.

Keywords: HER2neu; c-erbB-2; EGFR; HER1; Cervix cancer; Immunohistochemistry

Introduction

The epidermal growth factor receptor (EGFR) family comprises four structurally related transmembrane receptors: EGFR (HER1 or erbB-1), HER2neu (c-erbB-2), HER3, and HER4 [1–4]. In response to ligand specific binding, these receptors act by forming hetero- or homodimers and thereby initiate tyrosine kinase activity in the intracellular

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* Corresponding author. Department of Radiation Oncology, University of Utah, 50 North Medical Drive, Salt Lake City, UT 84132. Fax: +1-801-585-3502.
E-mail address: david.k.gaffney@hsc.utah.edu (D.K. Gaffney).

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domain. This amplification of tyrosine kinase activity results in the phosphorylation of several tyrosine residues in the C-terminus. These phosphorylated tyrosines then serve as binding sites for signal transducers and adapter molecules that initiate signaling pathways resulting in cell proliferation, differentiation, migration, adhesion, protection from apoptosis, and transformation [1,2,4].

The oncogenic pathway of some cells is thought to be initiated as a result of HER2neu and/or EGFR mutation, overexpression, structural rearrangements, and/or relief of normal regulatory or inhibitory pathways. The presence of EGFR and HER2neu receptors have also been associated with accelerated tumor progression and resistance to therapy for multiple types of malignancies [1,2,4]. 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.

HER2neu and EGFR expression have been investigated in the past by multiple methods. These include radioimmunoassays, immunohistochemical stains, enzyme immunoassays, and flow cytometry. Due to the inherent subjective nature of manual immunohistochemical stain interpretation, recently developed cellular imaging systems have been utilized to more objectively quantify staining intensity and distribution [5,6].

Few studies have evaluated the prognostic utility of multiple biomarkers in carcinoma of the cervix for patients treated in a homogeneous fashion (such as definitive radiotherapy alone). To our knowledge, this study is also the first comparative analysis of manual techniques and automated cellular imaging system techniques for the evaluation of HER2neu and EGFR immunohistochemical staining in carcinoma of the cervix. The goals of this study were to evaluate the correlation of HER2neu and EGFR expression with disease-free survival (DFS) and overall survival by both univariate and multivariate analysis, and secondarily to evaluate differences between manual interpretation and recently developed automated cellular imaging system (Chromavision Medical System).

Materials and methods

Fifty-five patients with FIGO stage IB–IVA carcinoma of the cervix on whom tumor tissue blocks were available were included in this study. Approval for this study was obtained from the LDS Hospital Institutional Review Board. Patients that received radiotherapy with definitive intent from 1981 to 1996 were included. Patients who received concurrent chemotherapy were also 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 were verified by one pathologist. Immunohistochemistry was performed for HER2neu and EGFR, and the expression of both biomarkers were quantitatively scored by ChromaVision cellular imaging technology for each patient (intensity scale based on pixel density, 0–4 for HER2neu and 0–16 000 for EGFR). Overexpression of HER2neu and EGFR were defined as >2.0 and >8000 (intensity scale), respectively. In addition, HER2neu and EGFR were manually scored for percent of positive cells (distribution): 0 = none, 1 ≤ 10%, 2 = 10–50%, 3 ≥ 50%; and for the intensity of staining with a subjective score of 0–3: 0 = no expression, 1 = minimal staining, 2 = moderate staining, 3 = heavy staining. The product of intensity and distribution (mean intensity) of staining was utilized for further statistical analyses. The location of EGFR staining was further described as membranous or cytoplasmic for each specimen.

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, immunohistochemical staining was performed with an automated immunohistochemical autostainer.

Detection for EGFR was performed with a secondary mouse anti-immunoglobulin linked to biotin following in-

Table 1
Patient and tumor characteristics ($N = 55$)

Characteristics	*N*	Median	SEM
FIGO stage			
IB	12		
II	22		
III	17		
IVA	4		
Histology			
Squamous	48		
Adenocarcinoma	5		
Adenosquamous	2		
Total dose to point A (cGy)		8038	250
Total treatment time (days)		44	1.6
# Brachytherapy insertions			
0	6		
1	36		
2	13		
Follow-up interval (months)			
All patients		24	7
Alive patients		69	15
Grade			
1	2		
2	34		
3	19		

SEM indicates standard error of the mean.

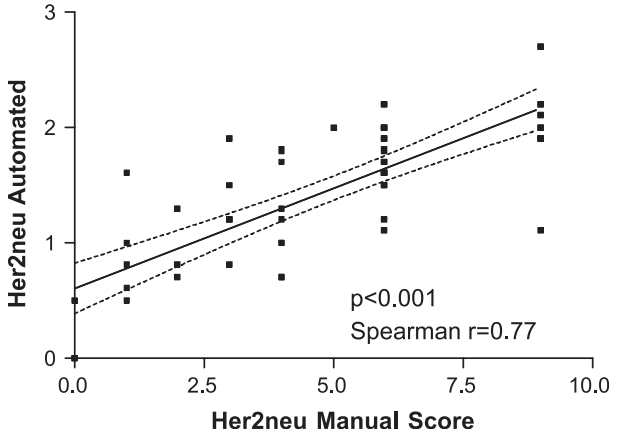

Fig. 1. Comparison of manual and automated methods for analysis of HER2neu expression in carcinoma of the cervix.

cubation with streptavidin linked to horseradish peroxidase. Color development was accomplished with diaminobenzidine as the chromagen. The EGFR detection kit was obtained from Dako (M3563) and was used in a concentration of 1:200. Detection for HER2neu was performed with a secondary rabbit anti-immunoglobulin linked to biotin following incubation with streptavidin linked to horseradish peroxidase. Color development for this assay was also accomplished with diaminobenzidine as the chromagen. The HER2neu detection kit was obtained from Dako (A0485) and was used in a concentration of 1:200.

Statistics

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 prognostic factors. The relationship between variables was assessed using Spearman's correlation. The Kaplan–Meier method was further 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% ($P < 0.05$).

Table 2
Correlations between clinical characteristics and biomarkers

	Spearman's *r*	*P* value
EGFR automated		
Clinical characteristics		
Histology	−0.29	0.035
Stage	0.15	0.28
Grade	−0.021	0.88
Biomarkers		
EGFR manual	0.64	<0.0001
EGFR membranous	0.41	0.002
EGFR cytoplasmic	−0.026	0.85
EGFR distribution	0.65	<0.0001
EGFR intensity	0.61	<0.0001
HER2neu automated	−0.23	0.089
HER2neu manual	−0.21	0.12
HER2neu automated		
Clinical characteristics		
Histology	0.28	0.038
Stage	−0.36	0.007
Grade	−0.12	0.40
Biomarkers		
EGFR manual	−0.28	0.04
EGFR membranous	−0.37	0.006
EGFR cytoplasmic	0.38	0.004
EGFR distribution	−0.29	0.035
EGFR intensity	−0.24	0.076
EGFR automated	−0.23	0.089
HER2neu manual	0.77	<0.0001

Results

Table 1 lists the pretreatment characteristics of this series of 55 patients with cervical cancer treated with definitive radiotherapy. Within this population, 48 patients had a pathologic diagnosis of squamous cell carcinoma, 5 had adenocarcinoma, and 2 had adenosquamous carcinoma. The median dose to point A was 8038 cGy.

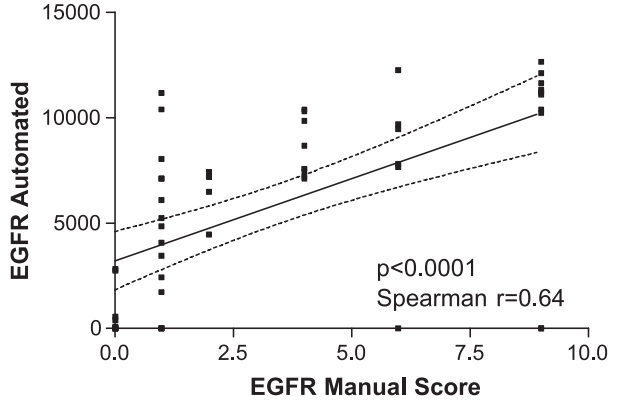

Fig. 2. Comparison of manual and automated methods for analysis of EGFR expression in carcinoma of the cervix.

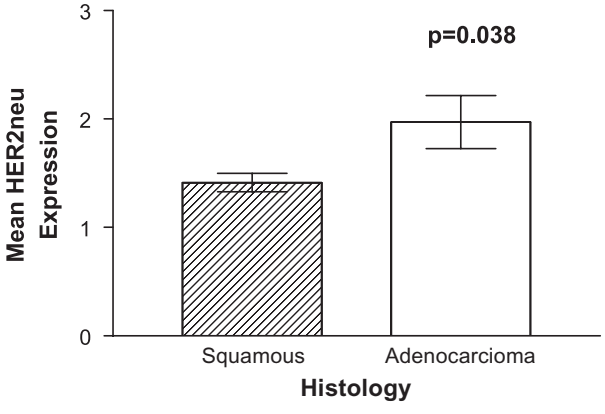

Fig. 3. Mean HER2neu expression by histologic subtypes of cervical carcinoma.

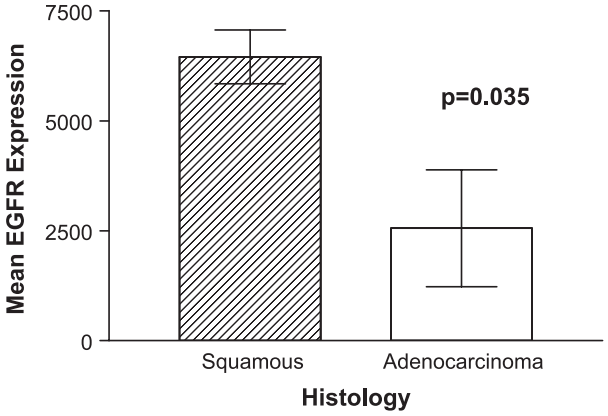

Fig. 4. Mean EGFR expression by histologic subtypes of cervical carcinoma.

Table 3
Prognostic factors: univariate analysis of clinical parameters and biomarkers with survival

Variable	Hazard ratio	95% CI	*P* value
Overall survival			
HER2neu automated	0.42	0.24–0.74	0.002
EGFR automated	1.67	0.80–3.50	0.175
EGFR membranous	1.94	1.02–3.69	0.043
Stage	11.70	2.57–53.34	0.001
Grade	0.83	0.11–6.43	0.85
Disease-free survival			
HER2neu automated	0.71	0.20–2.51	0.60
EGFR automated	4.58	0.82–25.45	0.08
EGFR membranous	4.18	1.02–17.05	0.05
Stage	3.97	0.71–22.14	0.12
Grade	0.81	0–8.36	0.96

Comparative analyses of the automated immunohistochemistry scoring system and manual scoring of HER2neu and EGFR were performed. Figs. 1 and 2 illustrate that statistically significant correlations were found between manually scored staining intensity of HER2neu and EGFR when compared with the automated cellular imaging/scoring system ($P < 0.001$ and $P < 0.0001$, respectively).

Patient and tumor characteristics were evaluated for significant correlations with each biomarker's expression (Table 2). In order to evaluate significant correlations between tumor histology and EGFR or HER2neu expression, patients were divided into squamous cell carcinoma and adenocarcinoma groups (including adenosquamous carcinoma). Increased expression of HER2neu correlated significantly with adenocarcinoma, and increased expression of EGFR correlated significantly with squamous cell carcinoma histology ($P = 0.038$ and $P = 0.035$, respectively) (Figs.

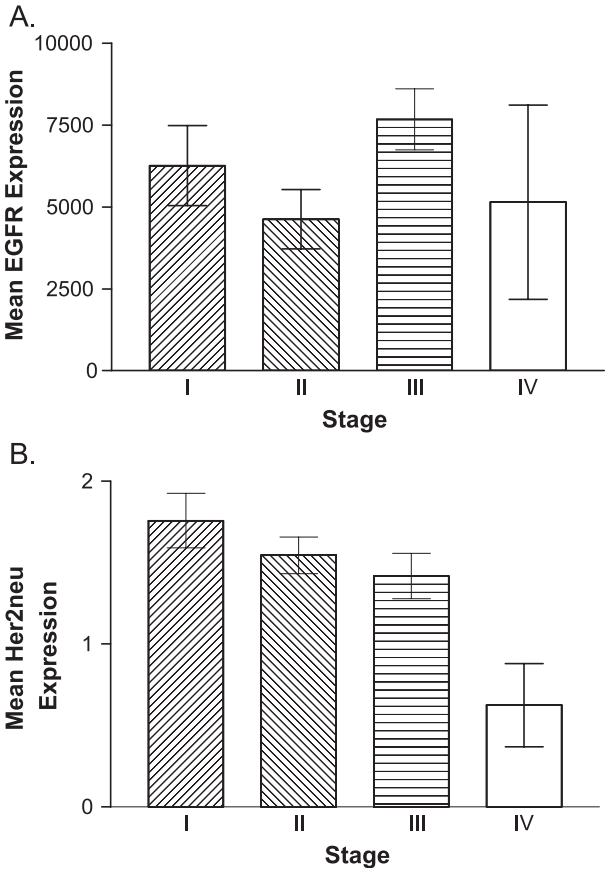

Fig. 5. Mean (A) EGFR and (B) HER2neu expressions according to initial tumor stage.

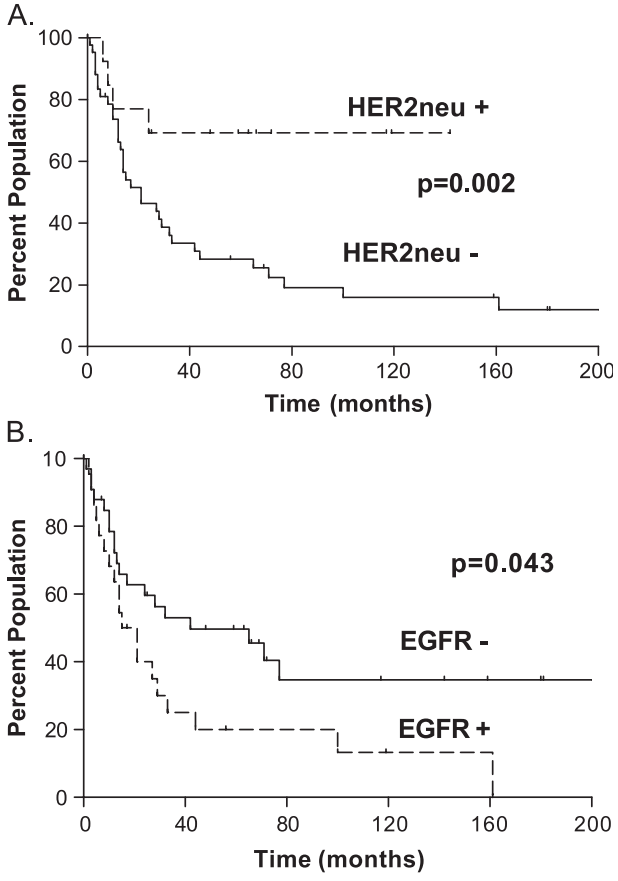

Fig. 6. Kaplan–Meier curves for overall survival related to (A) HER2neu expression and (B) EGFR expression in carcinoma of the cervix.

3 and 4). Inverse correlations were observed between HER2neu expression and clinical stage, EGFR membranous staining, and EGFR distribution ($P = 0.007$, $P = 0.006$, and $P = 0.034$, respectively). Fig. 5A illustrates statistically nonsignificant differences in mean EGFR expression between all stages of disease at presentation. In comparison (Fig. 5B), mean HER2neu expression was significantly elevated in early stage patients in our cohort.

Univariate (Table 3) and multivariate analyses were performed with overall survival (OS) and disease-free survival (DFS) as the primary endpoints. Increased FIGO stage, decreased HER2neu expression (intensity scale < 2.0), and increased membranous EGFR staining were significant for reduced OS on univariate analysis ($P = 0.001$, $P = 0.002$, and $P = 0.043$, respectively). Figs. 6A and B illustrate Kaplan–Meier curves for overall survival related to HER2neu and EGFR membranous expression. Membranous staining of EGFR was significant for diminished DFS on univariate analysis ($P = 0.05$). Multivariate analysis revealed only membranous staining of EGFR to be associated with both diminished DFS (HR = 4.18, 95% CI = 1.03–17.05, $P = 0.046$) and OS (HR = 3.53, 95% CI = 1.33–9.41, $P = 0.012$).

Discussion

HER2neu and EGFR are among biomarkers that have been investigated for prognostic merit in various malignancies, including carcinoma of the cervix. In the past, mixed results have been published as to the prognostic value of their expression. HER2neu and EGFR have both been shown in some studies to be independent predictors of a poor prognosis in carcinoma of the cervix [7–15]. On the other hand, other studies have also revealed no statistically significant correlation with an adverse outcome [16–20]. Mixed results have also been published on the prognostic significance of these markers in other gynecologic sites [21–26]. The conflicting results may be due to differences in institutional treatment standards and due to varied subjective interpretations of staining intensity and distribution between centers. Because staining intensity is judged on a continuum, differences in institutional "cut-off" for positive staining may also effect correlation with clinicopathologic results.

Two specific tumor-selective strategies that are based on the known functions of EGFR and HER2neu have been developed [1,2,4]. The first strategy involves development of humanized monoclonal antibodies against the receptor's extracellular domain, which 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 EGFR to transduce intracellular signals [1,2]. 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 include trastuzumab [27] (an anti-HER2neu antibody active in cases of breast cancer) and cetuximab (activity in colorectal cancer) [28]. In both instances, as predicted by preclinical data, there have been additive to super-additive effects with chemotherapy [29]. Tyrosine kinase inhibitors, such as ZD1839 (IressaTM) and erlotinib (OSI-774), have also demonstrated activity in early preclinical and clinical studies [30,31]. Further work in the area of gynecologic oncology is needed to elucidate the utility of these agents in carcinoma of the cervix.

It is noteworthy in our analysis that HER2neu overexpression was a favorable prognostic factor on univariate analysis for both OS and DFS, but was not significant on multivariate analysis. In previous studies, elevated HER2-neu was found to correlate with tumor size, local failure, and diminished survival in cervical cancer. Kihana et al. [13] found in their analysis 44 patients with cervical adenocarcinoma that increased expression of HER2neu correlated with a poorer prognosis. The absence of correlation between clinical prognosis and HER2neu expression was described by Ngan et al. [17]. Since HER2neu expression was increased in early stage tumors in our study (see Fig. 6), this confounding factor is likely the cause of loss of statistical significance of HER2neu on multivariate analysis in our study.

Although mixed results are evident in the literature, similar correlations of improved prognosis with decreased EGFR expression have been reported. Kim et al. [7] reported that overexpression of EGFR was associated with an impaired prognosis with respect to DFS and OS in carcinoma of the cervix. Kersemaekers et al. [10] also concluded from their analysis that the overexpression of EGFR was an independent predictor for poor prognosis in earlier stages (stages I and II) cervical cancer.

In this analysis, excellent correlation was found between manual scoring and automated immunohistochemical system grading of HER2neu and EGFR staining intensity and distribution. This ability to objectively obtain a score of intensity and distribution provides an unbiased approach to analysis of which results can than be correlated with clinicopathologic parameters and compared between institutions. As stated previously, the manual scoring of immunohistochemical staining intensity and distribution is a semiquantitative method with inter- and intrainstitutional variability. We feel that the continued development of automated immunohistochemistry tools will provide improved interdepartmental and interinstitutional correlation of results. Bishop et al. [5] reported that machine scoring of immunohistochemical stains is practical, rapid, and inherently reproducible. They also found in their analysis of HER2neu staining in breast carcinoma that automated scoring correlated highly with manual scores.

In conclusion, we found that increased expression of HER2neu identified patients with an improved DFS and OS

only on univariate analysis, and that multivariate analysis revealed only the membranous staining of EGFR to be associated with diminished OS. We also found a strong correlation of results between manually interpreted and automated immunohistochemical imaging systems. Important questions remain, such as (1) which tests in tumor tissue will best predict clinical response to HER2neu and EGFR inhibitors, (2) when in the tumors' natural history in which to intervene with receptor targeted drugs, (3) and the mechanism of acquired tumor cell resistance to these drugs. These questions and their implications may lead to tumor-specific antisignaling drug combinations in preneoplastic lesions and/or advanced cancers following the initial inhibition of HER2neu and EGFR at the tumor cell surface. These data support future investigation of the EGFR family of receptors and of medical therapeutics, which specifically target the expression of these proteins in carcinoma of the cervix.

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