Effect of Epidermal Growth Factor Receptor Expression Level on Survival in Patients with Epithelial Ovarian Cancer

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Abstract

Background: Several lines of laboratory evidence support the epidermal growth factor receptor (EGFR) as an adverse prognostic indicator in ovarian cancers. However, different methods of immunohistochemical assessment have yielded conflicting results. Here, we sought to determine the prognostic value of EGFR in ovarian cancer using a novel method of compartmentalized *in situ* protein analysis.

Methods: A tissue array composed of 150 advanced-stage ovarian cancers uniformly treated, with surgical debulking followed by platinum-paclitaxel combination chemotherapy, was constructed. For evaluation of EGFR protein expression, we used an immunofluorescence-based method of automated *in situ* quantitative measurement of protein analysis (AQUA).

Results: Mean follow-up time for the entire cohort was 34.4 months. Eighty-one of 150 cases had sufficient tissue for AQUA analysis. High tumor EGFR expression was associated with poor outcome for overall survival ($P = 0.0001$) and disease-free survival ($P = 0.0005$) at 3 years. In multivariable analysis, adjusting for well-characterized prognostic variables, EGFR expression status was the most significant prognostic factor for disease-free and overall survival.

Conclusion: The conflicting results in the literature regarding the prognostic value of EGFR may be due to the technical difficulties inherent in assessing EGFR with immunocytochemistry. In the present study, we show that measurement of EGFR protein levels in ovarian cancer using AQUA is feasible and can give important prognostic information.

Ovarian cancer is the fifth most common cancer in women. Despite the fact that it is highly curable if diagnosed early, cancer of the ovary kills more American women each year than all other gynecologic malignancies combined (1). There are no proven methods of prevention, and it often is a rapidly fatal disease. If diagnosed and treated while the disease is confined to the ovary, the 5-year survival rate is 95% (1); however, only about 29% of all cases are detected at this early stage.

The current management of patients with advanced disease (stages III and IV) involves optimal surgical debulking followed by chemotherapy. The current standard chemotherapeutic approach for ovarian cancer patients includes platinum-based (plus or minus taxanes) regimens. Although this treatment is highly effective, 60% to 80% of women still die of the disease (1). Traditional clinicopathologic factors do not accurately classify patients in relation to prognosis. The only validated marker for ovarian cancer is CA-125, which is detectable in the serum of >80% of women with ovarian carcinomas (2). However, CA-125 is reliable only in monitoring response to treatment or disease recurrence and not as a diagnostic or prognostic marker (3). Therefore, considerable interest lies in identifying molecular prognostic indicators to guide treatment decisions.

Several lines of evidence support the epidermal growth factor receptor (EGFR) as a molecular target for therapy in epithelial ovarian cancer. First, it has been shown that relative to normal ovarian epithelium, tissue extracts of over one third of ovarian carcinoma tissues have increased levels of factors that competed for binding ^{125}I-EGF to EGFR (4). Second, increased EGFR expression is observed in ~70% of ovarian carcinomas (5, 6). Furthermore, transfection of NIH:OVCAR-8 human ovarian carcinoma cells with an expression vector containing the human EGFR cDNA in an antisense orientation inhibited their invasive phenotype (7). Taken together, these findings indicate that EGF/ligand/EGFR axis is an important mechanism for supporting the autocrine growth of ovarian tumors.

A fundamental problem in EGFR-targeted therapy has been patient selection because the intensity of EGFR staining by immunohistochemistry has not been consistently associated with efficacy (8–10). The lack of association between EGFR levels and clinical outcome may be related to the nonquantitative nature of conventional immunohistochemistry. To overcome this problem, a method of automated quantitative analysis (AQUA), which provides precise, reproducible, measurement of antigen levels, free of the subjectivity associated

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with pathologist-based scoring, has been developed (11). AQUA provides continuous output scores, as opposed to the arbitrary nominal scores obtained with pathologist-based "by-eye" scoring of 0, 1, 2, or 3 or "positive" and "negative."

Here, we used AQUA on a tissue microarray composed of uniformly treated patients with epithelial ovarian cancer. Our study shows that measurement of EGFR protein levels on paraffin-embedded tissue using this method is feasible and provides important prognostic information.

Materials and Methods

Patient population. Inclusion criteria were primary epithelial ovarian cancer patients [Fesddration Internationale des Gynaecologistes et Obstetristes (FIGO) stages III and IV] who underwent surgical resection in the Department of Gynecology of Alexandra University Hospital in Athens between 1996 and 2003 and treated postoperatively with carboplatin and paclitaxel chemotherapy. In all cases, an effort was made for optimal surgical cytoreduction and adequate staging, which included, at least, total abdominal hysterectomy with bilateral salpingoophorectomy, inspection and palpation of all peritoneal surfaces and retroperitoneal area, biopsies of suspect lesions for metastases, infracolic omentectomy, and peritoneal washings. Included patients had stage III or IV disease according to the FIGO staging system. Grading was done by evaluation of tumor architecture, the amount of solid neoplastic areas, nucleus/cytoplasm ratio, and nuclear pleomorphism. The tumors were subdivided into three groups: well-differentiated (G_1), moderately differentiated (G_2), and poorly differentiated (G_3), according to these criteria.

Chemotherapy was instituted 2 to 3 weeks after surgery. All patients received platinum-paclitaxel chemotherapy. Gynecologic examination, CA-125 assay, and radiological investigations, if necessary, were done monthly for the clinical assessment of response, which was recorded according to WHO criteria (12). Follow-up examinations were done every month.

Tissue microarray construction. A tissue microarray consisting of tumors from each patient in the cohort was constructed at the Yale University Tissue Microarray Facility. Following institutional review board approval, the tissue microarray was constructed as previously described (13), including 150 cases. Tissue cores, 0.6 mm in size, were obtained from paraffin-embedded, formalin-fixed tissue blocks from the Alexandra University Hospital Department of Pathology archives. H&E-stained slides from all blocks were first reviewed by a pathologist to select representative areas of invasive tumor to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with 2-fold redundancy. Previous studies have shown that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for analysis by immunohistochemistry. Addition of a duplicate histospot, whereas not necessary, does provide marginally improved reliability (13). The tissue microarray was then cut to yield 5-μm sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

Quantitative immunohistochemistry. Tissue microarray slides were deparaffinized and stained as previously described (14). In brief, slides were deparrafinized with xylene followed by ethanol. Following rehydration in distilled water, antigen retrieval was accomplished by application of proteinase K and incubation for 30 minutes. Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 minutes. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin for 30 minutes at room temperature. Following these steps, slides were incubated with primary antibody at 4°C overnight. Primary monoclonal antibody to EGFR (clone H11, DAKO Corp., Carpinteria, CA) was used at 1:50 dilution in 0.3% bovine serum albumin/TBS. This antibody has been validated in previous studies using immunohistochemistry and Western blot analysis of normal and neoplastic tissue (15, 16). Subsequently, slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase–decorated dextran polymer backbone (Envision; DAKO) for 1 hour at room temperature. Tumor cells were identified by use of anti-cytokeratin antibody cocktail (rabbit anti-pancytokeratin antibody z0622, DAKO) with subsequent goat anti-rabbit antibody conjugated to Alexa546 fluourophore (A11035, Molecular Probes, Eugene, OR). We added 4′,6-diamidino-2-phenylindole to visualize nuclei. Target (EGFR) molecules were visualized with a fluorescent chromogen (Cy-5-tyramide; Perkin-Elmer Corp., Wellesley, MA). Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence. Slides were mounted with a polyvinyl alcohol–containing aqueous mounting media with antifade reagent (*n*-propyl gallate, Acros Organics, Vernon Hills, IL).

Automated image acquisition and analysis. Automated image acquisition and analysis using AQUA has been described previously (17, 18). In brief, monochromatic, high-resolution (1,024 × 1,024 pixel, 0.5 μm) images were obtained of each histospot. We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. 4′,6-Diamidino-2-phenylindole signal was used to identify nuclei, and the cytokeratin signal was used to define cytoplasm. Overlapping pixels [to a 99% confidence interval (99% CI)] were excluded from both compartments. The EGFR signal (AQUA score) was scored on a normalized scale of 1 to 255 expressed as pixel intensity divided by the target area. AQUA scores for each subcellular compartment (nuclear and cytoplasmic EGFR signal) were recorded. AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor.

Statistical analysis. Histospots containing <10% tumor as assessed by mask area (automated) were excluded from further analysis. AQUA scores represent expression of a target protein on a continuous scale from 1 to 255. It is often useful to categorize continuous variable to stratify patients into high versus low categories. Several methods exist to determine a cut point, including biological determination, splitting at the median, and determination of the cut point that maximizes effect difference between groups. If the latter method (the so-called "optimal *P*" approach) is used, a dramatic inflation of type I error rates can result (19). A recently developed program, X-Tile, allows determination of an optimal cut point while correcting for the use of minimum *P* statistics (20). As the AQUA technology is new, there are no established cut points available for quantitative EGFR expression. Therefore, for categorization of EGFR expression levels, the X-tile program was used to generate an optimal cut point. This approach has been successfully applied to AQUA data analysis (17). Two methods of statistical correction for the use of minimal *P* approach were used. First, the X-Tile program output includes calculation of a Monte Carlo *P* for the optimal cut point generated. Cut points that yield Monte Carlo *P*s < 0.05 are considered robust and unlikely to represent type I error. Second, the Miller-Siegmund minimal *P* correction referenced by Altman et al. was used (19). This approach is accepted in the statistical literature but relatively unknown in the medical/biological research community. Briefly, when making multiple comparisons to find the minimum *P* using the log-rank test, the false-positive rate (i.e., % number of times a marker that has no true prognostic value will be found to have a $P < 0.05$) can approach 40%. Altman's statistical adjustment generates a minimum *P* corrected to yield a true false-positive rate of 5%. The corrected *P* (P_{cor}) is calculated as follows: $P_{cor} = \phi(\zeta)\,[\zeta - (1/\zeta)]\log(e)\,[(1-\varepsilon)\langle 2\rangle/\varepsilon\langle 2\rangle] + 4\,\phi(\zeta)/\zeta$, where ϕ indicates the probability density function; P_{min} is the minimum *P* generated by evaluating multiple cut points; ζ is the $(1 - P_{min}/2)$ − quantile of the standard normal distribution; and ε denotes the proportion of values excluded from consideration as an optimal cut point. Our calculations were done using $\varepsilon = 0.10$. Disease-free survival and overall survival were subsequently assessed by Kaplan-Meier analysis with log-rank for determining statistical significance, and only P_{cor} was reported. This

approach has been successfully applied to AQUA data analysis (17). All survival analysis was done at 3-year cutoffs. CIs were assessed by univariate and multivariate Cox proportional hazards model.

Overall survival was defined as time from first day of chemotherapy to death from any cause. Disease-free survival was defined as time from first day of chemotherapy to the first of either death from any cause or disease progression (assessed by CA-125 increase and/or imaging studies). Performance status was dichotomized into "0" versus all others, and histologic type was dichotomized into serous versus all others. Although several cutoff values of residual volume tumor have been proposed, it has been reported that gradual gradations of residual disease can affect ovarian cancer prognosis. Our patient population was divided into two groups according to the extent of residual disease at first surgery: ≤2 cm and ≥2 cm. Comparisons of EGFR expression with FIGO stage and grade was made by Mantel-Haenszel χ^2 test. Comparisons of EGFR expression with performance status, histology, clinical response, and residual disease were made by Fisher's exact test. Comparison of EGFR expression status with age was made using Pearson correlation. All calculations and analyses were done with SPSS 12.0 for windows (SPSS, Inc., Chicago, IL).

Results

Clinical and pathologic variable analysis. One hundred fifty patients were included in the study. Mean follow-up time for the entire cohort was 34.4 months (range, 1-91.7 months). There were nine (6%) FIGO stage II, 108 (71.5%) stage III, and 33 (22.5%) stage IV. One hundred three (61%) patients had tumors of serous histology. Initial histologic grade was 14 well differentiated (9%), 49 moderately differentiated (33%), and 87 poorly differentiated (58%). One hundred three (61%) patients had tumors of serous histology. Following initial surgical debulking, residual disease by size was distributed as follows: 38 (25%) with <2 cm and 112 (75%) with >2 cm. For clinical response to initial therapy, complete response or partial response was recorded in 86 (57%) patients, and stable disease/no response was recorded in 64 (43%) patients. Demographic and clinicopathologic variables for the cohort are summarized in Table 1.

Quantitative immunohistochemistry for epidermal growth factor receptor protein expression and generation of optimal cut point by X-Tile analysis. Of the 150 patients included in this study, 81 (54%) had sufficient tissue for analysis of EGFR protein expression by AQUA. Tissues deemed insufficient had <10% tumor mask within the histospot, as represented on the tissue microarrays. As visualized by fluorescent immunohistochemistry, EGFR displayed predominantly strong membranous staining (Fig. 1A), whereas some tumors also displayed nuclear staining. Normalized AQUA scores were represented on a 1 to 255 scale. EGFR expression followed a skewed distribution as expected for

Table 1. Demographic, clinical, and pathologic data

Variable	*n*	*n* (with AQUA data)*	EGFR tumor expression class		*P*
			low	high	
Age (median)					
≤59	75	38	32	6	0.765
>59	75	43	36	7	
Differentiation					
Well	14	5	5	0	0.620
Moderate	49	30	25	5	
Poor	86	46	39	7	
Not recorded	1	1	1	0	
Initial histology					
Serous	103	56	46	10	0.325
All others	47	25	23	2	
FIGO stage					
II	9	6	6	0	0.550
III	108	54	45	9	
IV	33	21	18	3	
Residual disease (cm)					
≤2	38	21	20	1	0.170
>2	112	60	49	11	
Clinical response to chemotherapy					
PR + CR	86	48	40	8	0.753
All others	64	33	29	4	
Performance status					
No impairment	103	57	53	4	0.005†
All others	47	24	16	8	

Abbreviations: PR, partial remission; CR, complete remission.
*Eighty-one patients had sufficient tumor for AQUA analysis by 10% cut off point. Results do not include patients for whom AQUA or clinicopathologic information was not available.
†Significant at the 0.05 level.

a cancer tissue biomarker (Fig. 1B). Using the X-Tile program, an optimal cut point for tumor EGFR expression was determined at 61.97 AQUA units, with a Monte Carlo *P* of 0.0037 as determined by X-Tile. Monte Carlo *P*s less than $P = 0.05$ indicate robust and valid cut point selection. Patients with tumor EGFR expression of ≤61.97 were classified as low expressers ($n = 68$), and patients with EGFR expression of >61.97 were classified as high expressers ($n = 13$). Individual X-Tile analysis of nuclear and cytoplasmic EGFR levels showed optimal cut points; however, the Monte Carlo *P*s > 0.05 indicate lack of valid cut points.

Association of epidermal growth factor receptor expression and clinicopathologic variables. Patients with low EGFR expression levels were more likely to have excellent performance status ($P = 0.005$). Otherwise, there was no association between EGFR staining and clinicopathologic variables, including age, response to chemotherapy, histologic type, histologic grade, FIGO stage, and residual disease (Table 1).

Univariate survival analysis. Tumor AQUA expression level of EGFR was examined for association with 3-year overall survival and disease-free survival using Kaplan-Meier survival analysis with log-rank statistic for determining significance. As use of an optimized cut point can result in increased type I error, the Miller-Siegmund correction method was applied to all Kaplan-Meier analyses. Kaplan-Meier survival curves generated for tumor EGFR, high versus low expression, are given in Fig. 2. High tumor EGFR expression was associated with poor outcome for overall survival ($P = 0.0001$) and disease-free survival ($P = 0.0005$). Patients with high tumor EGFR expression had 25% disease-free and 33% overall survival compared with 34.8% and 71% for patients with low tumor EGFR expression ($P_{cor} = 0.0005$ and 0.0001, respectively). Results for univariate Kaplan-Meier analysis of EGFR expression and survival are summarized in Table 2.

Multivariable survival analysis. Using the Cox proportional hazards model, we did multivariable analysis to assess the predictive value of tumor EGFR expression. Tumor EGFR expression by AQUA was analyzed for overall survival and disease-free survival. We also included the following known prognostic variables in the regression model: FIGO stage, grade, residual disease, response to chemotherapy, and initial histology. High tumor EGFR level (99% CI, 3.26-24; $P = 0.0001$) along with FIGO stage (99% CI, 1.11-5.04; $P = 0.026$) were

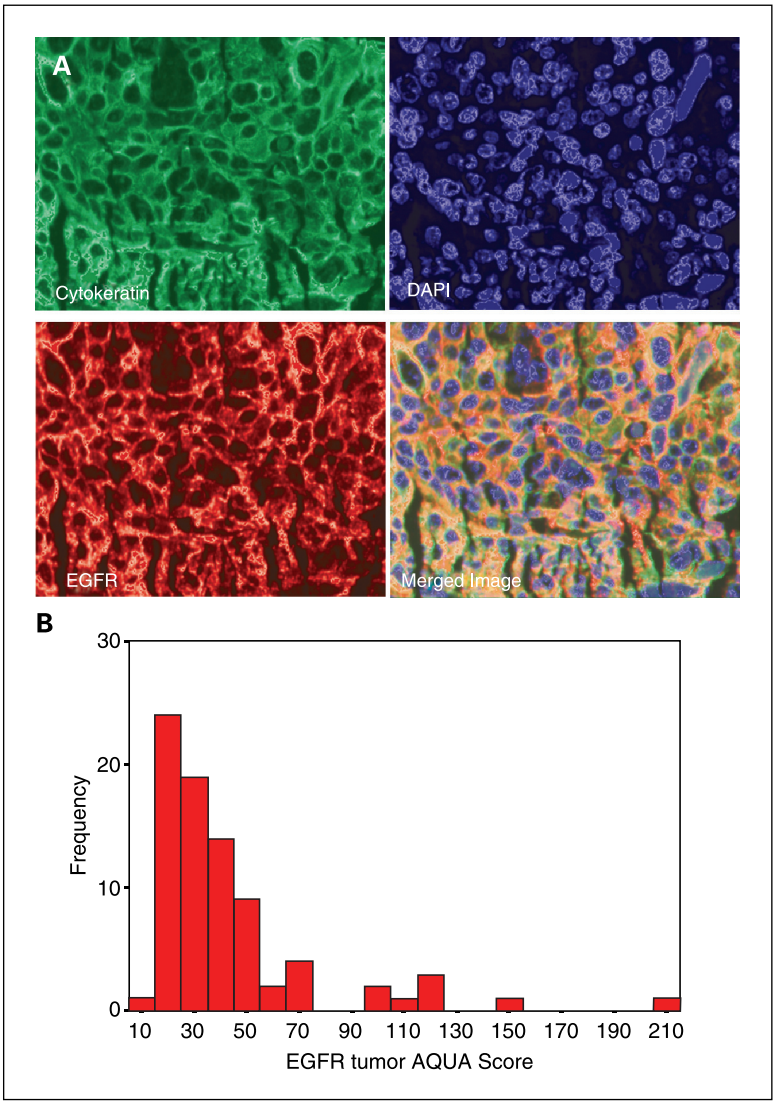

Fig. 1. *A,* protein expression of EGFR was determined using a novel method of protein analysis based on immunofluorescence. Digital images of each tumor spot were captured using Cy3 anti-cytokeratin antibody to generate a tumor mask. 4′,6-Diamidino-2-phenylindole was used to visualize nuclei, and Cy5 was used to visualize EGFR. A three-merged color image for each tumor was generated. *B,* AQUA analysis showed a left-skewed distribution for EGFR tumor score as expected for a biomarker.

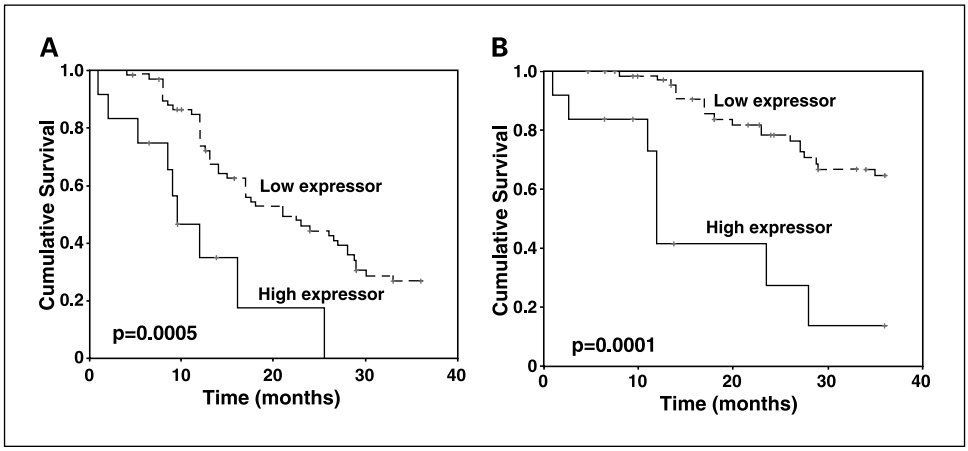

Fig. 2. Kaplan-Meier survival analysis for disease-free survival (*A*) and overall survival (*B*) by tumor EGFR expression level. Patients with high tumor EGFR AQUA score had worse disease-free survival and overall survival.

significant predictor variables of overall survival. For disease-free survival, residual disease (99% CI, 1.44-9.91; $P = 0.007$) and tumor EGFR (99% CI, 1.64-8.65; $P = 0.002$) were significant predictors. Results of multivariable survival analyses are summarized in Table 3.

Discussion

In the present study, using quantitative immunohistochemistry, we were able to show that EGFR expression levels are inversely correlated with outcome in epithelial ovarian cancer. In multivariable analysis, adjusted for well-recognized prognostic indicators, EGFR maintained its independent prognostic value. Several investigators have studied the association of EGFR expression by conventional immunohistochemistry with outcome in ovarian cancer. EGFR overexpression rates in these studies cover a wide range (19-77%) probably due to different methods of pathologist-based scoring (21–24). Previous results on the prognostic role of EGFR are likewise conflicting. EGFR has in some studies been associated with decreased survival in univariate analysis, but no additional prognosticeffect was found after adjustment for the classic prognostic factors (21, 22, 25, 26). On the contrary, Skirnisdottir et al. (24) proved EGFR and grade to have prognostic effect in multivariate analysis.

Our analysis shows the power of continuous automated assessment to define subclasses of tumors not achievable using standard pathologist-based assessment. Using this technology, we were able to show an association between EGFR expression levels and outcome consistent with the biological role of EGFR in tumor behavior. AQUA has been validated as an *in situ* proteomic technique in multiple tumor types where we were able to show associations between biomarker levels and outcome not discernable with the standard pathologist-based scoring (11, 18).

With the availability of EGFR inhibitors, the need for assays that will appropriately select patients for EGFR-targeted therapy becomes more urgent. Studies evaluating EGFR protein expression in tumor tissues have used several methods and, in general, have provided a rather loose definition of overexpression without an accurate determination of receptor levels. Studies with cetuximab and the EGFR tyrosine kinase inhibitor gefitinib have shown responses in human tumors and cell lines expressing a wide range of EGFR levels from very low to very high (27). One implication of these data is that low EGFR-expressing cells but still inhibitor-sensitive cells may not score as positive with the widely used immunohistochemical method. An important limitation with standard immunohistochemistry is that low antibody concentrations lack sensitivity at the low end of protein expression, and high antibody concentrations fail to differentiate between mid and high levels of protein expression because of saturation combined with higher background and nonspecific staining. Moroni et al. (28) analyzed EGFR in patients with colorectal cancer and found

Table 2. Univariate 3-year survival analysis (Kaplan-Meier log-rank)

Variable	Mean survival (mo)	% Cumulative survival (95% CI)	*P*
Disease-free survival			
High* tumor EGFR	12	25 (18-45)	0.0005†
Low tumor EGFR	22	34.8 (31-44)	
Overall survival			
High tumor EGFR	17	33.3 (22-47)	0.0001‡
Low tumor EGFR	31	71 (62-88)	

*The cut off point of high tumor EGFR is based on X-tile analysis, which generated optimal cut off point of 61.97 AQUA units.
†Significant at the 0.05 level.
‡Significant at the 0.01 level.

Table 3. Multivariate 3-year survival analysis by Cox regression

Variable	Hazard ratio (95% CI)	*P*
Disease-free survival		
Initial histology	1.396 (0.73-2.66)	0.311
FIGO stage	1.725 (0.97-3.08)	0.065
Residual disease	3.776 (1.44-9.91)	0.007*
Clinical response to chemotherapy	0.752 (0.51-1.11)	0.15
Grade	0.596 (0.35-1.01)	0.055
High EGFR tumor expression	3.761 (1.64-8.65)	0.002*
Overall survival		
Initial histology	1.930 (0.79-4.72)	0.149
FIGO stage	2.36 (1.11-5.04)	0.026†
Residual disease	2.39 (0.71-8.03)	0.159
Clinical response to chemotherapy	1.899 (0.75-4.81)	0.176
Grade	0.494 (0.23-1.04)	0.064
High EGFR tumor expression	8.862 (3.26-24.06)	0.0001*

*Significant at the 0.01 level.
†Significant at the 0.05 level.

that 90% of those who responded to humanized EGFR antibody cetuximab (C225) had an increased number of gene copies of EGFR. An increase in copy number should lead to higher EGFR protein levels. Therefore, it seems paradoxical that EGFR protein levels by immunohistochemistry do not correlate with response to EGFR-targeted therapies. However, as previously mentioned, conventional immunohistochemistry is a nonquantitative method and, therefore, inadequate to provide an accurate assessment of EGFR protein levels. A discrepancy between gene amplification rate and protein overexpression assessed by immunohistochemistry providing discordant prognostic information has also been reported with cyclin D1 in head and neck cancers (29). Another plausible explanation for the discordant prognostic information provided by immunohistochemistry and fluorescence *in situ* hybridization is that protein overexpression may also occur via unknown mechanisms, which precede gene amplification, such as translocations, inversions, or yet unknown causes of transcriptional activation. A comparison of the incidence of EGFR overexpression by AQUA with that of gene amplification by fluorescence *in situ* hybridization is being undertaken in our laboratory.

Our finding of EGFR nuclear staining also deserves mention. EGFR is generally known as plasma membrane receptor tyrosine kinase, which sends signals to the nucleus via the mitogen-activated protein kinase, the phospholipase C/protein kinase C, and the phosphatidylinositol 3-kinase pathways. However, recently, data are accumulating to imply that nuclear localization and action of EGFR may occur as well (30, 31). EGFR may enter the nucleus and directly act as transcriptional factor, bypassing the protein phosphorylation cascades. Lin et al. (30) showed that nuclear EGFR is associated with the promoter region of cyclin D1 *in vivo* and activates transcription. Nuclear localization and action of EGFR are worthy of study, as they constitute a potential mechanism of resistance to EGFR-targeted therapies. Because nuclear EGFR directly activates transcription bypassing the protein phosphorylation cascades, EGFR-rich tumors may not respond to EGFR inhibitors blocking only receptor-mediated signaling.

In conclusion, in the present study, we show that measurement of EGFR protein levels in ovarian cancer is feasible and can give important prognostic information. AQUA may prove to be a useful technology in pharmacodynamic studies to identify patient cancers sensitive to EGFR inhibitors.

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