

Overexpression of *HER-2/neu* Is Associated with Poor Survival in Advanced Epithelial Ovarian Cancer¹

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ABSTRACT

Previous studies have suggested that overexpression of *HER-2/neu* oncogene occurs in 15–40% of breast cancers and that overexpression is associated with poor prognosis. In the present report, we have used an immunohistochemical technique involving a monoclonal antibody specifically reactive with the external domain of *HER-2/neu* to study expression of *HER-2/neu* in frozen sections of normal ovary and advanced epithelial ovarian cancer. The intensity of staining for *HER-2/neu* was always moderate or less (0–2+) in normal ovarian epithelium. Among 73 ovarian cancers, 50 (68%) had staining similar to that for normal ovarian epithelium (0–2+) while 23 (32%) stained heavily (3+). Survival of the 23 patients with high *HER-2/neu* expression (median, 15.7 months) was significantly worse ($P = 0.001$) than that of the 50 patients (median, 32.8 months) with normal *HER-2/neu* expression. In addition, patients whose tumors had high *HER-2/neu* expression were significantly less likely to have a complete response to primary therapy ($P < 0.05$) or have a negative second-look laparotomy when serum CA 125 levels were normal preoperatively ($P < 0.05$). These findings suggest that *HER-2/neu* deserves further evaluation as a prognostic marker in epithelial ovarian cancer.

INTRODUCTION

The human *HER-2/neu* gene (*c-erbB-2*) encodes a cell surface glycoprotein that is similar in structure to the epidermal growth factor receptor (*c-erbB*) (1). Like the epidermal growth factor receptor, the *HER-2/neu* gene product has been shown to be a transmembrane protein that includes a cysteine rich extracellular ligand binding domain, a hydrophobic membrane spanning region, and an intracellular tyrosine kinase domain (2). Due to these structural similarities to the epidermal growth factor receptor, it has been postulated that the *HER-2/neu* gene product may function as a receptor for some as yet unidentified growth factor. In normal tissues, *HER-2/neu* expression has been demonstrated predominantly in epithelial cells (3); however, the physiological function of *HER-2/neu*, like that of other known peptide growth factor receptors, has not been clarified.

The *HER-2/neu* gene first was identified as the oncogene associated with the development of neuroblastomas in rats exposed to ethylnitrosourea *in utero* (4). In this animal model, it has been shown that *HER-2/neu* is oncogenic due to a single point mutation in the membrane spanning region (5). In human malignancies, however, *HER-2/neu* amplification and overexpression rather than point mutation have been noted. In breast cancer, several studies have suggested that overexpression of *HER-2/neu* occurs in 15–40% of cancers (6–10). Some,

but not all, of these studies also have suggested that overexpression of *HER-2/neu* is associated with poor survival (6, 10). In addition, it has been shown that *HER-2/neu* sometimes is amplified and overexpressed in other human malignancies including ovarian cancer (11). Most recently, it has been suggested that, as in breast cancer, overexpression of *HER-2/neu* in ovarian cancer might be associated with poor survival (12).

In the present study, we have utilized a murine monoclonal antibody specifically reactive with the external domain of *HER-2/neu* (13) to study expression of *HER-2/neu* in normal and malignant ovarian tissues using immunohistochemical techniques. The intensity of staining for *HER-2/neu* in patients with advanced epithelial ovarian cancer was compared to that of normal ovarian epithelium. We examined the relationship between the level of *HER-2/neu* expression and prognostic factors such as age and histological grade. In addition, we examined the relationship between the level of *HER-2/neu* expression and response to primary therapy, findings at second-look laparotomy, and survival.

MATERIALS AND METHODS

Patients. All of the patients in this study with ovarian cancer underwent exploratory laparotomy in conjunction with treatment for International Federation of Gynecologists and Obstetricians stage III or IV epithelial ovarian cancer (14) at Duke University between 1985 and 1989. During this time, fresh tissue from 81 patients was frozen in liquid nitrogen. In 8 cases, however, the tissue samples were found to be inadequate for immunohistochemical analysis due to extensive necrosis or a paucity of tumor cells in the specimen. Among the 73 remaining patients, tumor was obtained at the initial debulking operation in 50 cases. Among these 50 patients, 5 also had tumor samples frozen at second-look or subsequent laparotomy. In an additional 23 cases, tumor was obtained only at second-look laparotomy or at the time of recurrence. In addition, samples of normal ovary were frozen from 6 patients undergoing hysterectomy and bilateral salpingo-oophorectomy for benign gynecological disease.

All histological material from the 50 cases in which tumor was obtained at initial surgery was reviewed by a single pathologist (A. K.) and the histological type (serous, mucinous, clear cell, endometrioid, undifferentiated) and histological grade (well differentiated, moderately differentiated, poorly differentiated) were determined using the WHO criteria (15). Most of the other 23 patients in whom tumor was obtained at a subsequent operation had undergone initial surgery elsewhere and slides were not available for review. In these 23 patients, however, the diagnosis of epithelial ovarian cancer was confirmed by review of material obtained at surgical surveillance procedures.

Immunohistochemistry. All tissue samples were snap frozen and stored at -70°C until analyzed. Tissue samples were frozen in Tissue Tek OCT compound (Ames Division, Miles Laboratories, Elkhart, IN) and 4–6- μm -thick cryosections were mounted on gelatin coated slides. The slides were air dried overnight at room temperature to enhance the cellular morphology. The slides were fixed in acetone for 10 min at room temperature and then air dried again for 30 min. Then the slides were washed in phosphate buffered saline three times for 5 min.

Immunohistochemical staining was performed using the Elite Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Slides were

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placed in a humidified chamber and incubated for 15 min with several drops of a 1:200 dilution of horse serum in phosphate buffered saline with 2% bovine serum albumin. Then, 70 μ l of primary antibody were applied over the tissue sections for 60 min. After washing with phosphate buffered saline, biotinylated goat anti-mouse IgG antibody was added for 30 min followed by the avidin-peroxidase complex. Finally, the slides were developed for 4 min with the enzyme substrate diaminobenzidine (0.5% diaminobenzidine in 0.05% Tris buffer-0.6% hydrogen peroxide). The slides then were rinsed for 10 min in running tap water, counterstained with 1% methyl green (Sigma Chemical Co., St. Louis, MO), and dehydrated and mounted.

Monoclonal Antibodies. Purified TA1 is a murine monoclonal antibody that was generated by immunization of mice with cells expressing the human *HER-2/neu* molecule (Applied bioTechnology Inc., Cambridge, MA) (13). This antibody is not cross-reactive with the epidermal growth factor receptor. We tested TA1 at concentrations of 0.01–5.0 μ g/ml and determined that maximal immunohistochemical staining for *HER-2/neu* was obtained with a concentration of 0.5 μ g/ml. This concentration of antibody subsequently was used to stain all of the tissue samples in this study. We used purified mouse IgG specific for nonhuman tissue (Coulter Immunology, Hialeah, FL) at a dilution of 1:100 as a negative control. Anti-keratin AE1/AE3 (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used as a positive control.

Immunohistochemical localization of *HER-2/neu* was evaluated using serial sections. First, a section stained with hematoxylin and eosin was examined to evaluate the histology. Then, a negative control slide stained with nonspecific mouse IgG was examined to assess nonspecific staining. Next, a positive control slide stained with anti-keratin antibody was examined to confirm the presence of viable tumor cells in the section. Cases in which the negative and positive controls were not adequate were repeated. Finally, the slide in which TA1 was used as the primary antibody was examined. The intensity of staining for *HER-2/neu* was evaluated by two observers using a double headed microscope. The identity of the patients was not known to the observers while the slides were being scored. The intensity was graded as 0 (staining not greater than negative control), 1+ (light staining), 2+ (moderate staining), or 3+ (heavy staining). After all of the cases had been scored over the course of several weeks, all of the cases were rescored in 1 day. In seven cases (9.6%), there was a difference in intensity of one category between the two scores assigned. In these cases, the latter score was used since, on that occasion, the intensity of staining of all the cases was compared and contrasted directly.

Statistics. Tables were analyzed using Fisher's two tailed exact test. Survival estimates were calculated using the Kaplan-Meier life table method (16). Differences in survival were tested with the log rank statistic (17).

RESULTS

Immunohistochemically detectable *HER-2/neu* protein was noted in normal ovarian epithelium in 5 of 6 cases examined. In the 5 cases in which staining was seen, every epithelial cell was stained. In 3 cases, light staining was noted while in 2 cases moderate staining was seen (Fig. 1). In all cases, adjacent ovarian stroma did not express *HER-2/neu*.

Among the 50 patients with advanced ovarian cancer in whom tumor was frozen at initial surgery, 39 patients had serous tumors, 4 patients had mucinous tumors, 1 patient had an endometrioid tumor, 1 patient had a clear cell tumor, 4 patients had undifferentiated tumors, and 1 patient had a mixed serous/clear cell tumor. The histological grade of these 50 cancers was: 4 cases, well differentiated; 20 cases, moderately differentiated; 26 cases, poorly differentiated.

Among these 50 cases of ovarian cancer, in which tissue was frozen at initial surgery, 33 (66%) were found to stain for *HER-2/neu* with similar intensity as was seen in normal ovarian epithelium (0–2+). Among these 33 patients, 10 had no staining, 10 stained lightly, and 13 stained moderately. In the



Fig. 1. Moderate (2+) staining for *HER-2/neu* in normal ovarian epithelium.

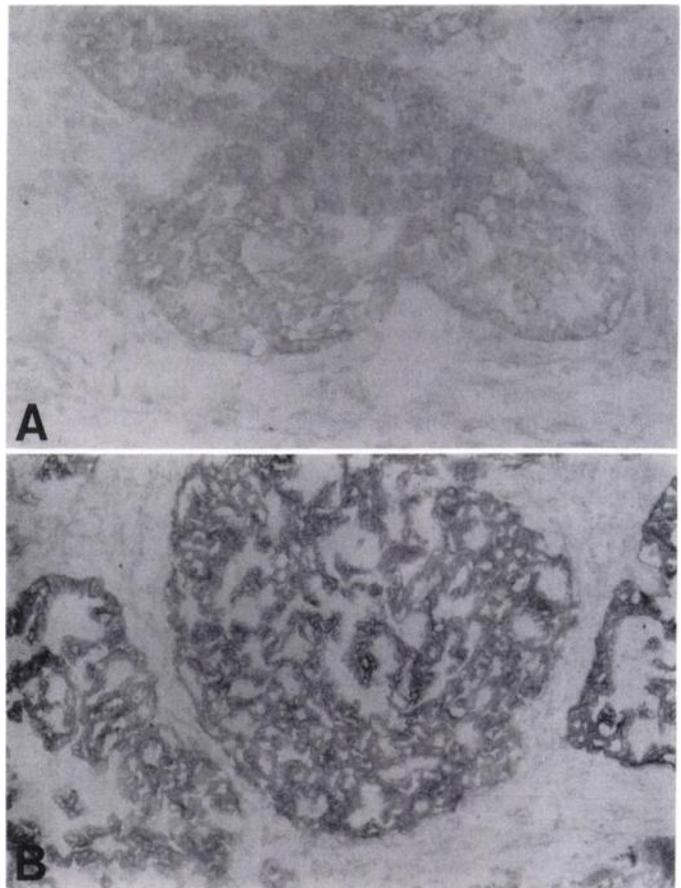


Fig. 2. *HER-2/neu* expression in ovarian cancer. A, light (1+) staining for *HER-2/neu*; B, heavy (3+) staining for *HER-2/neu*.

cancers, like normal ovarian epithelium, when staining was present, it was confined to the epithelial component. No staining was seen in the surrounding stroma. In contrast, 17 ovarian cancers (34%) were found to stain heavily for *HER-2/neu*. In Fig. 2, examples of ovarian cancers with normal and heavy staining for *HER-2/neu* are demonstrated. Among these 50 patients, 13 (26%) were considered optimally debulked (maximal diameter of largest residual tumor < 1 cm). No relationship was found between debulking status and *HER-2/neu* expression. Thirty-eight % of optimally debulked patients had heavy staining for *HER-2/neu* compared to 33% of patients who were suboptimally debulked. Among the 13 optimal patients, how-

ever, 6 of 8 with normal *HER-2/neu* expression remain alive compared to only 1 of 5 with high *HER-2/neu* expression.

In 11 of the 50 patients in whom tumor was obtained at the time of initial cytoreductive surgery, *HER-2/neu* expression was evaluated in both the primary tumor and 1 to 3 metastases. In 10 of these cases, within a single patient the same intensity of staining for *HER-2/neu* was seen in the primary tumor and metastases. In one case, bilateral ovarian tumors were present and one stained lightly while the other stained heavily. In addition, in five patients, *HER-2/neu* expression was evaluated in tumor obtained both at initial surgery and at a subsequent laparotomy. In all five cases, the level of *HER-2/neu* expression was unchanged following therapy.

Since *HER-2/neu* expression did not appear to be altered during the course of treatment, we also analyzed *HER-2/neu* expression from 23 patients in whom tumor was available from second-look or other subsequent laparotomy. In 17 of these 23 cases (74%), *HER-2/neu* expression was similar to that of normal ovarian epithelium (0–2+). In 6 cases (26%) *HER-2/neu* expression was stronger than that of normal ovarian epithelium (3+). This frequency of high *HER-2/neu* expression is similar to that which was found in the 50 cases in which tumor was obtained at initial laparotomy (34%).

Overall, 23 of 73 patients (32%) were found to have high *HER-2/neu* expression. The median age of the patients with high *HER-2/neu* expression (63.5 years) was somewhat higher than that of patients with normal *HER-2/neu* expression (58.8 years), but the difference was not statistically significant. We found no significant relationship between histological grade and high *HER-2/neu* expression among the 50 patients in whom the slides were available for review. In addition, we found no relationship between histological grade and survival, although there were only four patients with well differentiated cancers.

Table 1 examines the relationship between the level of *HER-2/neu* expression and response to primary therapy in all 73 patients in this study. Among the 50 patients with normal *HER-2/neu* expression, 12 (24%) did not undergo second-look laparotomy due to patient refusal or poor medical condition compared to 6 of 23 patients (26%) with high *HER-2/neu* expression. In the group with normal *HER-2/neu* expression, four patients did not undergo second-look due to clinically obvious progression of disease as was the case in six patients with high *HER-2/neu* expression. Table 1 demonstrates that a significantly higher proportion of evaluable patients with normal *HER-2/neu* expression achieved a surgically documented complete response relative to those with high *HER-2/neu* expression ($P < 0.05$). Among 38 evaluable patients with normal *HER-2/neu* expression (34 second-look and 4 progression of disease prior to second-look), 12 achieved a negative second-look (32%). In contrast, among 17 evaluable patients with high *HER-2/neu* expression (11 second-look and 6 progression of disease) only one (6%) achieved a negative second-look. Among the 12 patients with normal *HER-2/neu* expression who had a negative second-look laparotomy, 4 patients (33%) subsequently have developed recurrent disease. The other 8 patients

Table 1 Relationship between the level of *HER-2/neu* expression and response to primary therapy

<i>HER-2/neu</i> expression	Unevaluable patients	Progression of disease	Second-look positive		Second-look negative
			Gross	Microscopic	
Normal (0–2+)	12	4	17	5	12
High (3+)	6	6	8	2	1

Table 2 Relationship of *HER-2/neu* expression to findings at second-look laparotomy in patients with normal serum CA 125 levels

<i>HER-2/neu</i> expression	Second-look findings	
	Persistent cancer	Complete response
Normal (0–2+)	10	12
High (3+)	5	0

who have not developed recurrent disease have a median survival of 34 months. The one patient with high *HER-2/neu* expression who had a negative second-look laparotomy also developed recurrent disease.

Among the 45 patients who underwent second-look laparotomy, 39 had serum CA 125 levels measured preoperatively. In 12 of these cases, the serum CA 125 level was elevated (>35 units/ml). As expected, all of these patients were found to have persistent disease at second-look laparotomy. Twenty-seven patients had a normal serum CA 125 level prior to second-look laparotomy. Table 2 demonstrates that, among these 27 patients, there was a significantly greater probability of finding persistent disease at second-look in patients whose tumors had high *HER-2/neu* expression compared to those whose tumors had normal *HER-2/neu* expression ($P = 0.05$). Among patients with normal *HER-2/neu* expression, 45% were found to have persistent disease at second-look despite a normal serum CA 125 level. In contrast, all 5 patients with high *HER-2/neu* expression (100%) had persistent disease at second-look despite a normal serum CA 125 level.

In Fig. 3, the relationship between *HER-2/neu* expression and survival is demonstrated. Among the 50 patients with normal *HER-2/neu* expression (0–2+), 21 are dead of disease, 14 are alive with disease, and 15 are alive with no evidence of disease. In contrast, among the 23 patients with high *HER-2/neu* expression (3+), 20 are dead of disease, 1 is dead of intercurrent disease, 2 are alive with disease, and none is alive with no evidence of disease. The median survival of the 50 patients with normal *HER-2/neu* expression was 32.8 months (95% confidence intervals, 27–49 months). The median survival of 23 patients with high *HER-2/neu* expression was 15.7 months (95% confidence intervals, 12–17 months). Among the entire group of 73 patients in the study (Fig. 3A), Kaplan-Meier life table survival of the group with high *HER-2/neu* expression was significantly worse than that of the group with normal *HER-2/neu* expression ($P < 0.001$). A similar survival curve which includes only the 50 patients in whom tumor was obtained at initial surgery (Fig. 3B) also reveals significantly worse survival in the group with high *HER-2/neu* expression ($P < 0.001$).

DISCUSSION

The finding that abnormalities in growth factor receptor expression often accompany malignant transformation has led several groups to study the role of individual growth factor receptors and their respective oncogenes in various human malignancies. For example, several investigators have noted that some squamous cancers express increased numbers of epidermal growth factor receptors relative to normal squamous cells (18). In addition, others have reported that only a proportion of breast cancers express cell surface epidermal growth factor receptors and that expression of receptor is associated with poor prognosis (19). We and others, however, have not observed a correlation between epidermal growth factor receptor expression and survival in other cancers including lung and endometrial adenocarcinomas, although as in breast cancer,

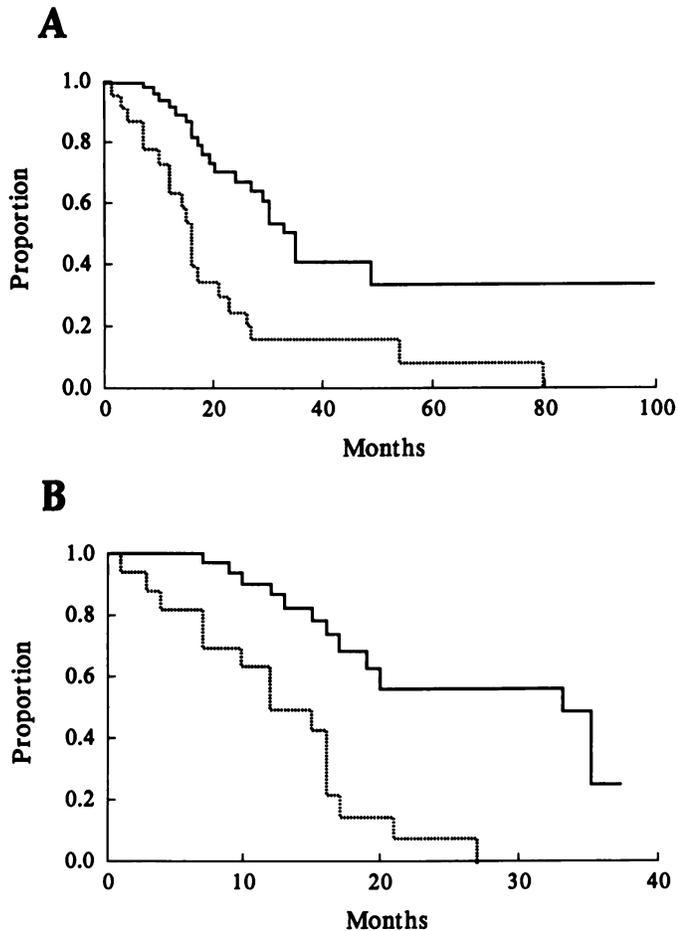


Fig. 3. Relationship between the level of *HER-2/neu* expression and survival in ovarian cancer. *A*, all 73 patients ($P < 0.001$); *B*, 50 patients in whom tumor was obtained at initial surgery ($P < 0.001$). —, normal *HER-2/neu* expression (0–2+); ·····, high *HER-2/neu* expression (3+).

only a proportion of cancers appear to express receptor (20).

Although the role of *HER-2/neu* as a growth factor receptor remains speculative, overexpression of *HER-2/neu* has been reported to occur in approximately 15–40% of breast cancers (6–11). In contrast, *HER-2/neu* overexpression has been found to be a relatively infrequent event in most other human malignancies (21). In breast cancer, it has been shown that overexpression of *HER-2/neu* usually is associated with *HER-2/neu* gene amplification (10–30%), although approximately 10% of breast cancers may have *HER-2/neu* overexpression in association with a normal *HER-2/neu* gene copy number (12). In some but not all studies, patients in whom *HER-2/neu* was overexpressed were found to have a significantly poorer survival than those with normal *HER-2/neu* expression (6, 10, 12).

In addition to breast cancer, overexpression of *HER-2/neu* has been reported more recently in a significant proportion of cases of ovarian cancer (12). It also has been suggested that, as in breast cancer, *HER-2/neu* overexpression is found in ovarian cancers with extremely aggressive biological behavior. The results of the present study are in agreement with these findings in several respects. First, we found high levels of *HER-2/neu* expression in approximately one-third of ovarian cancers relative to normal ovarian epithelium. Second, high *HER-2/neu* expression was associated with poor outcome in this study. Among the 73 patients in this study, only 15 (21%) are alive without evidence of disease. All 15 of these patients had normal *HER-2/neu* expression. The association of *HER-2/neu* over-

expression with poor prognosis was noted in both optimally and suboptimally debulked patients.

In this study, we also demonstrated for the first time a relationship between *HER-2/neu* expression and response to primary therapy and findings at second-look laparotomy. Patients with normal *HER-2/neu* expression had a 5-fold higher likelihood of achieving a surgically documented complete response to primary therapy relative to patients with high *HER-2/neu* expression. Furthermore, among patients with a normal serum CA 125 level at second-look laparotomy, residual cancer was found in all 5 patients with high *HER-2/neu* expression compared to only 45% of patients with normal *HER-2/neu* expression.

These findings suggest that assessment of *HER-2/neu* expression at the time of initial surgery allows identification of a subset of patients with a particularly poor prognosis. Although currently no effective therapy exists for these patients, they could be observed more closely for signs of progression of disease during primary therapy and then entered into experimental protocols sooner while tumor burden was still relatively small.

Prior studies in breast cancer have shown that the level of *HER-2/neu* expression in a given patient is the same in the primary tumor and lymph node metastases (7, 22). Similarly, in ovarian cancer, we found that the level of *HER-2/neu* expression in peritoneal metastases usually resembled that seen in the primary tumor. In addition, *HER-2/neu* expression did not appear to be altered following chemotherapy since the level of *HER-2/neu* expression was consistent between tumor obtained at initial surgery and that at subsequent laparotomy. Thus, it appears that, in most cases, the level of *HER-2/neu* expression can be assessed using an adequate sample of tumor regardless of the site or operation from which the tumor is obtained. In a single patient in whom bilateral ovarian cancers were present, however, high *HER-2/neu* expression was noted in one tumor while normal *HER-2/neu* expression was noted in the other. This may be a reflection of multifocal carcinogenesis, which has been proposed in the past as an explanation for bilateral tumors.

If *HER-2/neu* expression is confirmed as an important independent prognostic variable in breast and ovarian cancer, further studies will be needed to determine which method for measuring *HER-2/neu* expression is most practical in clinical practice. In the present study, we used an immunohistochemical technique to compare the level of *HER-2/neu* expression in frozen ovarian cancer specimens with that found in normal ovarian epithelium. The advantage of the immunohistochemical method is that it allows direct observation of *HER-2/neu* expression at a cellular level. Other methods in which tissue homogenates are used to measure levels of DNA, RNA, and protein all are subject to error due to differences in the proportion of cancer cells relative to stromal elements in tumor samples. On the other hand, scoring of *HER-2/neu* expression by immunohistochemical assessment is less easily reproducible. In this study, we found that in 9.6% of cases there was a disagreement within one category when the intensity of staining for *HER-2/neu* was scored twice. Presently, however, efforts are under way to develop a computerized image analysis program that can quantitate immunohistochemical staining of *HER-2/neu*. This technology, which already has been applied successfully to immunohistochemical analysis of estrogen receptor and progesterone receptor (23), hopefully will increase the objectivity and reproducibility of immunohistochemical determination of *HER-2/neu* expression. Finally, the effect of

formalin fixation and paraffin embedding on *HER-2/neu* antigenicity must be clarified further. If antigenicity is altered by such processing, as often is the case, then quantitative assessment of *HER-2/neu* expression on routinely processed tissue will not be possible. In fact, this has been proposed as an explanation for why some studies of *HER-2/neu* expression in archival material have failed to find a correlation between the level of *HER-2/neu* expression and survival in breast cancer (7, 9).

Although *HER-2/neu* first was found to be oncogenic due to a point mutation rather than overexpression (5), More recently, it has been shown that overexpression of the normal *HER-2/neu* gene product *in vitro* also can elicit malignant transformation (24). In addition, it has been shown that binding of an anti-*HER-2/neu* antibody to *HER-2/neu*-transformed NIH 3T3 cells is followed by down-regulation of cell surface *HER-2/neu* levels (25). In this system, down-regulation of *HER-2/neu* was associated with a decreased ability to sustain anchorage independent growth, one of the primary characteristics that distinguish cancer cells from normal cells. These findings further strengthen the hypothesis that *HER-2/neu* overexpression in breast and ovarian cancer is closely related to the aggressive biological behavior of this subset of cancers. Further studies are needed to clarify the role of *HER-2/neu* in the regulation of growth of normal and malignant cells.

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