ANNALS OF SURGERY
Vol. 221, No. 6, 677–684

The Significance of c-*erb* B-2 and p53 Immunoreactivity in Patients with Adenocarcinoma of the Esophagus

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Objective

Studies in breast cancer suggest that p53 and c-*erb* B2 protein overexpression are predictive of outcome. The authors determined whether these molecular markers correlated with treatment response and survival in patients with adenocarcinoma of the esophagus and esophagogastric junction.

Method

Immunostaining for p53 and c-*erb* B2 was performed on paraffin-embedded specimens from 42 patients with esophageal adenocarcinoma. All patients received neoadjuvant chemotherapy (cisplatin and fluorouracil [5-FU] × 3 cycles) and irradiation (4500 rads) followed by resection.

Results

In this cohort of patients, 79% (33/42) were positive for p53, and 43% (18/42) were positive for c-*erb* B2. p53 positivity correlated with residual disease in the resection specimen but not with disease-free survival. Although c-*erb* B2 negativity correlated with residual disease after resection and a 5-year survival of 10%, c-*erb* B2 positivity was associated with a 5-year actuarial survival of 60%.

Conclusions

Although p53 protein overexpression is commonly observed in adenocarcinoma of the esophagus, its prognostic value appears limited. In contrast, c-*erb* B2 protein expression predicts a favorable response to therapy and improved survival.

The incidence of adenocarcinoma of the esophagus and esophagogastric junction is rising faster than any cancer in the United States.[1] Despite recent advances in our understanding of the molecular biology, pathogenesis, and treatment of this disease, prognosis remains dismal, with a 5-year survival of less than 10%.[2] Thus, for the majority of patients with esophageal cancer, the mainstay of treatment is palliation.

Recent studies have examined p53 and c-*erb* B2 (HER2/*neu*) protein overexpression as prognostic markers in breast cancer. In a study of women with stage I and II breast cancer, Marks et al. reviewed 230 paraffin-

Presented at the 106th Annual Session of the Southern Surgical Association, December 4–7, 1994, Palm Beach, Florida.
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Accepted for publication January 18, 1995.

embedded specimens immunostained for p53 and c-*erb* B2.[3] They observed that p53 protein overexpression was associated with a significantly shorter overall survival whereas c-*erb* B2 expression correlated with a shorter overall and disease-free survival.[3] Furthermore, on the basis of multivariate analysis, p53, c-*erb* B2, and nodal status each demonstrated independent prognostic value.[3] In the Cancer and Leukemia Group B randomized adjuvant-chemotherapy study (CALGB 8541), 442 tissue blocks taken from women with node-positive breast cancer were immunostained for c-*erb* B2.[4] These investigators found a significant dose-response effect of adjuvant chemotherapy in patients with overexpression of c-*erb* B2.[4] Thus, p53 and c-*erb* B2 proteins may be useful molecular markers to predict outcome in breast cancer and to identify patients most likely to benefit from adjuvant chemotherapy.

Because of the considerable morbidity attributed to chemoradiation and surgery and its uncertain value in many patients with adenocarcinoma of the esophagus, the identification of molecular markers that accurately predict treatment response and survival would be of obvious clinical benefit. Those patients with responsive tumors could then be identified prospectively and considered for combined chemoradiation and surgery. Thus, those patients predicted to respond poorly could be spared the significant morbidity, time, and financial expense of chemotherapy, irradiation, and radical resection. Instead, these subjects may constitute a group of patients ideally suited for alternative treatment strategies or palliative measures alone. In this report, we retrospectively examined whether p53 or c-*erb* B2 protein overexpression in archival tissue specimens correlated with treatment response and outcome in patients with adenocarcinoma of the esophagus and esophagogastric junction.

MATERIALS AND METHODS

Of 154 patients with adenocarcinoma of the distal esophagus and gastroesophageal junction treated at Duke University Medical Center between 1985 to 1994, 73 completed a course of preoperative chemotherapy and irradiation, followed by esophagogastrectomy. In 42 of the 73 patients, tissue blocks were available and adequate for immunohistochemical analysis. A detailed description of the neoadjuvant chemoradiation protocol used in this study has been reported previously.[5,6] Briefly, patients with adenocarcinoma of the esophagus received intravenous infusions of cisplatin, 20 mg-m^{-2}-day^{-1}, and fluorouracil (5-FU), 1000 mg-m^{-2}-day^{-1}, for 5 days. After the first course of chemotherapy, patients were discharged from the hospital and readmitted after 3 to 4 weeks. During the second course of chemotherapy, concurrent radiotherapy was given 5 days per week for 5 weeks, for a total of 4500 rads. During the last week of radiotherapy, the final course of chemotherapy was given. On completion of the neoadjuvant protocol, all subjects were restaged. For those patients considered inoperable because of distant metastases or patient refusal, radiation treatment was extended to 6000 rads.

Antibodies

As previously described,[3] c-*erb* B2 overexpression was detected in paraffin-embedded sections using Triton pAB-2 (Triton Diagnostics, Alameda, CA) at a concentration of 1 μg/mL. This affinity-purified rabbit polyclonal antibody reacts specifically with the intracellular domain of the p185 c-*erb* B2 protein. In matched frozen tissues, the mouse monoclonal antibody, Tab #251 (provided by B. Langton, Triton Biosciences, Alameda, CA), was used at a concentration of 2.5 μg/mL. Tab #251 is an immunoglobulin G_1 (IgG1) antibody that reacts with an epitope in the extracellular portion of the receptor. PAb1801 (Oncogene Science, Manhassett, NY) was used to detect the p53 protein in paraffin-embedded tissues at a concentration of 1 μg/mL. PAb1801 is an IgG_1 mouse monoclonal antibody that recognizes a denaturation-resistant epitope in the p53 protein between amino acids 32 and 79. Normal rabbit serum and mouse IgG_1 (Gibco, Grand Island, NY) were used to assess nonspecific staining in serial sections. In all cases, antibodies were diluted before use in primary antibody diluting buffer (Biomeda Corp., Foster City, CA).

Immunohistochemical Analysis

Paraffin blocks were cooled in an ice water bath for 30 minutes before sectioning. Sections measuring 4 to 6 μm thick were cut and placed on Plus slides (Baxter, McGraw Park, IL). The sections were air dried for 15 minutes, heat-fixed to the slide at 42 C, and air dried overnight at room temperature. The slides were stored at room temperature until use. To detect c-*erb* B2 in paraffin-embedded tissues, an alkaline phosphatase technique was used. Sections were first deparaffinized in xylene and then hydrated. After hydration, slides were rinsed three times for 5 minutes in TRIS-buffered saline (pH 7.6) followed by a 20-minute incubation in 10% normal goat serum diluted in TRIS-buffered saline. After rinsing, the slides were incubated with the diluted primary antibody (pAB-1) at 4 C for 18 hours in a humidified chamber. The slides were rinsed in TRIS-buffered saline and a 1:1000 dilution of biotinylated goat antirabbit IgG (Jackson Immuno Research, West Grove, PA) was applied for 35 minutes at room temperature. After this step, an alkaline phosphatase-conjugated streptavi-

din diluted 1:300 was applied for 30 minutes. The slides were washed in TRIS-buffered saline and developed in the CAS Red enzyme substrate solution as directed by the manufacturer (CAS, Elmhurst, IL). After chromogen development, the slides were washed in running tap water for 10 minutes and counterstained with 1% methyl green. The slides were dehydrated in acetone, cleared in xylene, and mounted in permanent coverslipping medium. The procedure to detect p53 in paraffin has been described previously,[3] with the following modifications: after deparaffinization, endogenous peroxidase activity was eliminated by treating the slides with EtOH-H_2O_2 (45 mL 95% EtOH: 3 mL 30% H_2O_2) for 10 minutes at 42 C. The sections were heated in a 700-watt microwave oven in 0.1 mol/L citrate buffer for 5 minutes. The slides were cooled at room temperature for 3 minutes, and the microwave heating was repeated. Slides were then allowed to come to room temperature for 30 minutes. This antigen-retrieval process significantly enhanced the staining intensity. Slides were preincubated in normal horse serum, and the remainder of the procedure was completed as described previously.[3] Immunohistochemical staining was scored by one of us (MRG) in a blinded fashion. A similar method was used for the scoring of all p53 staining.

Statistical Analysis

Survival was calculated by the Kaplan-Meier method considering treatment-related deaths and deaths caused by esophageal cancer. Comparisons were made by the log-rank method. Univariate analyses were performed using the χ^2 test. The null hypothesis was rejected at the $\alpha = 0.05$ significance level.

RESULTS

Demographics and Oncogene Expression

In this cohort of 42 patients, mean age was 61.5 ± 10.2 years, 40 (95%) patients were men, and 41 (98%) were white. In Table 1, the mean age and distribution by sex and race are displayed as a function of c-*erb* B2 and p53 protein overexpression. No significant differences were observed in the age, sex, and race of patients with c-*erb* B2 and p53 positive and negative tumors. The prevalence of overexpression of p53 protein was 79% (33 of 42); and of c-*erb* B2 protein was 43% (18 of 42).

Pathology and Oncogene Expression

Thirty-two tumors (76%) were located at the esophagogastric junction, nine in the lower third (21%) of the esophagus, and one in the middle third (2%). The distribution of tumors as a function of oncogene protein expression is shown in Table 1; no significant differences were observed between groups. Barrett's metaplasia was observed in 22 of 42 (52%) specimens. The frequency of c-*erb* B2 and p53 positivity occurred equally in the presence or absence of Barrett's metaplasia (Table 1). Lymph node metastases were found in 14 of 42 (33%) surgical resection specimens. Neither c-*erb* B2 nor p53 protein overexpression correlated with the presence of lymph node metastases; however, the relationship between lymph node metastases and p53 protein overexpression exhibited a trend toward significance ($p = 0.11$; Table 1).

Response to Neoadjuvant Therapy and Actuarial Survival

The combination of chemotherapy and radiation is capable of eradicating all histologic evidence of malignancy in resected esophageal specimens.[6] In the entire series of 73 patients who underwent preoperative chemotherapy and irradiation followed by esophagogastrectomy, no residual cancer was found in the resection specimens of 18 (25%). The overall survival of patients who achieved complete sterilization of the esophagus was superior to those patients with residual microscopic disease (Fig. 1). The sterilized group attained a 5-year actuarial survival of 60%, whereas no patient with residual microscopic disease survived beyond 50 months ($p = 0.001$).

Of the 42 patients in the present study, 10 (24%) had complete sterilization of the esophagus. In Table 1, the presence or absence of residual microscopic disease after neoadjuvant therapy was related to oncogene overexpression. Both c-*erb* B2 negativity and p53 positivity were significantly correlated with the presence of residual microscopic disease after neoadjuvant therapy (Table 1).

Prognostic Significance of c-*erb* B2 and p53 Staining

In this cohort of patients, actuarial survival curves were calculated on the basis of c-*erb* B2 status (Fig. 2). Median follow-up was 11.6 months (range 2.4–87.0 months). The median survival of patients whose tumors lacked c-*erb* B2 protein overexpression was 13.6 months, whereas those with c-*erb* B2 protein overexpression demonstrated a 5-year actuarial survival of 55% (Fig. 2). Unlike c-*erb* B2, the influence of p53 overexpression on survival failed to reach statistical significance (Fig. 3). The inter-relationship between c-*erb* B2 and p53 status is depicted in Figure 4. The combination of c-*erb* B2 positivity and p53 negativity correlated with improved survival in our study. In particular, survival was superior compared with patients with c-*erb* B2 negative/p53 pos-

Table 1. RELATIONSHIP BETWEEN P53, C-*ERB* B2 STAINING, AND CLINICAL PARAMETERS

Parameter	c-erb B2 (−)	c-erb B2 (+)		p53 (−)	p53 (+)	
N	24	18		9	33	
Age	61.8 ± 10.3	61.0 ± 10.0		57.9 ± 9.2	62.5 ± 10.2	
Sex	23M:1F	17M:1F		9M:0F	31M:2F	
Race						
White	24	17		9	32	
Black	0	1	*NS*	0	1	*NS*
Tumor site						
Middle/3	1	0		0	1	
Lower/3	5	4		1	8	
GEJ	18	14	*NS*	8	24	*NS*
Barrett's metaplasia						
Present	14	8		4	18	
Absent	10	10	*NS*	5	15	*NS*
Lymph nodes metastases						
Present	9	5		1	13	
Absent	15	13	*NS*	8	20	*NS*
Pathology						
Sterile	3	7		5	5	
Residual disease	21	11	$p < 0.05$	4	28	$p = 0.01$

itive ($p = 0.0001$), or c-*erb* B2 negative/p53 negative ($p = 0.005$) tumors.

DISCUSSION

Although most patients with carcinoma of the esophagus have regional or distant metastases and a dismal prognosis, a small subset of patients respond favorably to neoadjuvant therapy followed by esophagectomy. Beginning in the early 1980s, all patients with carcinoma of the esophagus treated at Duke have been considered for enrollment in a prospective clinical trial using neoadjuvant chemotherapy (cisplatin and 5-FU × 3 cycles) and irradiation (4500 rads) followed by esophagogastrectomy.[5,6] After operation, all surgical specimens were examined carefully for evidence of residual tumor within the resected esophagus and periesophageal soft tissue. Those specimens characterized by complete absence of residual microscopic disease were classified as sterile. We observed that after chemotherapy and irradiation, sterilized specimens occurred in 40% of patients with squamous-cell carcinoma and 20% with adenocarcinoma.[6]

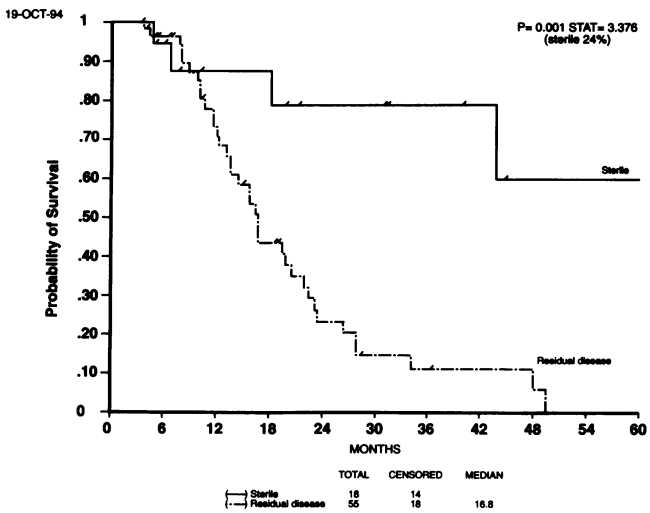

Figure 1. The survival of 73 consecutive patients where comparison between those with residual disease and sterile specimen is plotted over a 5-year period.

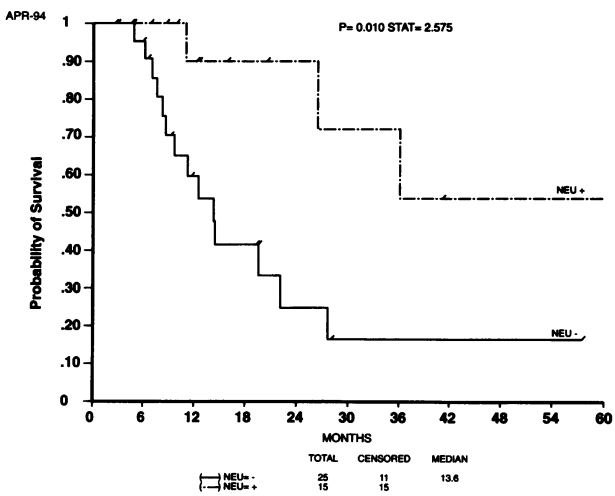

Figure 2. The difference in survival between those patients with c-*erb* B2 (neu) positive and those patients with c-*erb* B2 negative tumors.

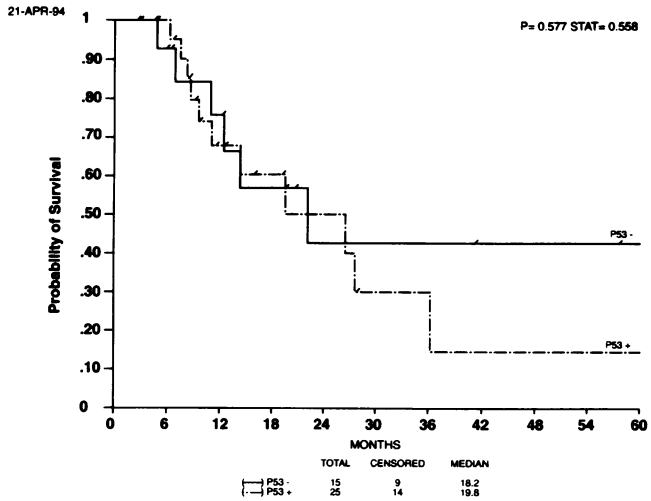

Figure 3. Survival of patients with p53 negative *vs.* those who were p53 positive.

Other investigators have reported similar rates of sterilized resection specimens using cisplatin and 5-FU in conjunction with external-beam irradiation, ranging from 15% to 56% (mean 30%).[7-14] Moreover, the presence of a sterilized resection specimen correlated with prolonged survival in these patients.[6,10,14-16] In our experience with squamous-cell carcinoma of the esophagus, the overall 5-year actuarial survival in the sterilized group was 40% *versus* 10% in the group with residual microscopic disease.[6] With adenocarcinoma, the 5-year survival in the sterilized group was 60% *versus* 10% in the group with residual disease.[6] Thus, histopathologic sterility of the resected esophagus and periesophageal soft tissue after neoadjuvant therapy is predictive of a superior outcome after resection in patients with esophageal cancer.

Widespread use of neoadjuvant chemotherapy and irradiation for the treatment of esophageal cancer is limited by its potential toxicity. In our experience, these toxicities commonly include mild to moderate radiation-induced esophagitis, infection, and significant myelosuppresion in 50% of patients—with 20% unable to tolerate the final cycle of chemotherapy because of profound leukopenia.[17] Furthermore, in a recent multivariate risk factor analysis, radiation doses exceeding 50 Gy were found to be independent predictors of perioperative mortality.[17] In a study by Poplin and the Southwest Oncology Group (SWOG), moderate to severe nausea and vomiting were experienced in 33%, mucositis in 11%, leukopenia in 31%, and life-threatening myelosuppression in 8% of patients with esophageal cancer treated with neoadjuvant cisplatin and 5-FU and irradiation.[14] Because of the potential toxicities associated with chemotherapy and irradiation, the low rate of complete histologic response, and the conspicuous absence of randomized clinical studies affirming its value, it is doubtful that all patients with esophageal cancer benefit from adjuvant therapy before operative resection. Thus, the identification of molecular markers that will accurately predict those tumors responsive to adjuvant therapy would be of enormous clinical benefit.

Overexpression of p53 protein is commonly present in esophageal cancer. In this cohort of patients with adenocarcinoma of the esophagus, the prevalence of p53 protein expression was 80%. This correlation has been observed by others.[18-21] Jankowski et al. found p53 protein overexpression in 7 of out 15 cases (53%) of esophageal adenocarcinoma.[21] In a similar immunohistochemical study, Flejou et al. stained frozen sections of malignant esophageal tumors using a monoclonal antibody directed against wild type and mutated p53 protein (PAb 1801).[18] They observed p53 protein expression in 8 of 11, or 73%, of esophageal adenocarcinomas.[18] Mutation and overexpression of p53 protein is a common genetic alteration observed in adenocarcinoma of the esophagus.

Although p53 protein overexpression is commonly observed in adenocarcinoma of the esophagus, its prognostic value appears limited. In this study, survival curves computed on the basis of p53 positivity and negativity were not significantly different. However, p53 positivity was strongly predictive of residual disease in the esophageal specimen after adjuvant therapy. This apparent discrepancy may reflect the small number of patients in the study (only nine were p53 negative), or a type II statistical error. Indeed, in related immunohistochemical studies on breast cancer[3] and on carcinoma of the lung,[22] p53 protein expression was demonstrated to correlate with a shorter disease-free survival. Alternatively, the putative relationship between p53 protein overexpression and decreased survival may have been con-

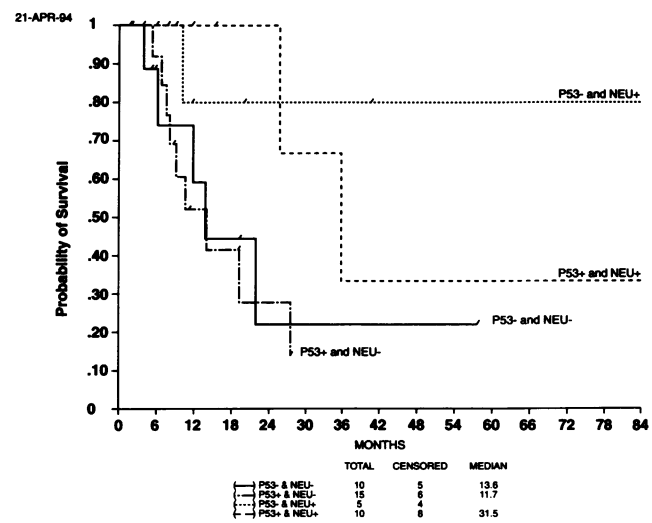

Figure 4. The survival between the patients who were p53 negative and *cerb* B2 (neu) positive and the other groups.

founded by the beneficial effects of neoadjuvant chemotherapy and irradiation on the natural course of the disease. Thus, failure to observe a significantly shorter disease-free survival in patients with p53 positive tumors may in fact reflect on the ability of neoadjuvant therapy to improve it. This is supported by the superior 5-year survival achieved in this study compared with recent series for esophagogastrectomy alone. In the absence of an appropriate control group, however, this possibility remains speculative.

Nearly one half of all patients with adenocarcinoma of the esophagus overexpressed c-*erb* B2 protein in this series. Although c-*erb* B2 protein overexpression has been well described in adenocarcinomas of the breast, ovary, and stomach, few studies have investigated this dominant oncogene in esophageal cancer. In an immunohistochemical study of 15 resected esophageal adenocarcinomas, Jankowski et al. found positive staining for c-*erb* B2 protein in 11, or 73%.[21] Moreover, these authors reported positive immunostaining in 9 of 15 (60%) specimens of benign Barrett's metaplasia, and 2 of 15 (13%) specimens of normal gastric mucosa.[21] The differential expression of c-*erb* B2 in adenocarcinoma of the esophagus, benign Barrett's metaplasia, and normal gastric epithelium suggested to these authors that c-*erb* B2 protein expression was a marker for malignant potential.[21] Because the prevailing model of carcinogenesis in Barrett's epithelium entails a continuous sequence of histopathologic change from metaplasia to dysplasia to invasive carcinoma, quantitative expression of c-*erb* B2 may parallel such histologic change.

Overexpression of c-*erb* B2 protein is predictive of improved survival in patients with adenocarcinoma of the esophagus. Although the fundamental mechanism underlying this observation is beyond the scope of the present study, the observed correlation between c-*erb* B2 negativity and residual microscopic disease suggests that c-*erb* B2 protein expression may be a marker for susceptibility to chemotherapy and irradiation. In the recently reported Cancer and Leukemia Group B randomized adjuvant-chemotherapy study (CALGB 8541), tumors from women with node-positive breast cancer were immunostained for c-*erb* B2.[4] A significant dose-response effect of adjuvant chemotherapy (cyclophosphamide, doxorubicin, and 5-FU) was observed in patients with overexpression of c-*erb* B2.[4] Although these investigators speculated that c-*erb* B2 expression may indicate increased susceptibility to higher doses of doxorubicin, this agent was not used in our chemotherapeutic regimen. On the other hand, both protocols included 5-FU. An alternative explanation for the beneficial impact on survival is that c-*erb* B2 overexpression may simply denote a less aggressive tumor phenotype. In a study of 120 patients with gastric adenocarcinoma, Motojima et al. showed that well-differentiated adenocarcinomas had a higher prevalence of c-*erb* B2 expression than poorly differentiated carcinomas.[23] Although the present study failed to demonstrate a significant correlation between c-*erb* B2 protein overexpression and a lower incidence of regional lymph node metastases, other phenotypic characteristics (e.g., tumor depth of invasion) warrant further investigation.

Although most patients with adenocarcinoma of the esophagus have a poor prognosis, a small subset of patients respond favorably to neoadjuvant therapy followed by esophagectomy. Widespread use of neoadjuvant chemotherapy and irradiation for the treatment of esophageal cancer, however, is limited by its potential toxicity. Thus, molecular markers that will accurately identify those tumors responsive to adjuvant therapy are of obvious clinical benefit. Although p53 protein overexpression is commonly observed in adenocarcinoma of the esophagus, its prognostic value appears limited. In contrast, overexpression of c-*erb* B2 is predictive of improved survival after combined modality therapy. Further studies are necessary to elucidate the relationship between the increase in c-*erb* B2 protein expression and the susceptibility to 5-FU in adenocarcinoma of the esophagus.

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Discussion

DR. SUSAN GALANDIUK (Louisville, Kentucky): Thank you, Dr. Jurkiewicz, Dr. Copeland, Ladies and Gentlemen. This paper and the one following it both describe the use of chemoradiation preoperatively in esophageal carcinoma and are particularly exciting because of very poor 5-year survival rate in these patients.

I have five questions for the authors.

First, on the choice of cisplatin. As you know, it is a very cytotoxic drug with a lot of systemic toxicity. In other cancers, for example, anal cancers or gynecological cancers, mitomycin-C or hydroxyurea have been used with success. Have they tried any other agents?

Then, a second question regarding staging information. There was no mention made of staging of these patients, and one would expect that both staging and tumor differentiation would very much predict and influence the effect of chemoradiation preoperatively. Was there any correlation between p53 and c-*erb* B-2 expression and the stage of these tumors?

Then, thirdly, there has been a lot of criticism of the technique of immunohistochemistry in determining the expression of protein both as to reliability and reproducibility. Have the authors tried to correlate immunohistochemistry results with those obtained using fresh tissue for determination of protein marker expression, or even used PCR techniques to examine gene expression in these biopsy samples?

Since EGF receptor is so structurally similar to c-*erb* B-2, have they measured the EGF receptor expression in these biopsy specimens or looked at other bad preoprostic markers in breast carcinoma, for example, Cathepsin D?

The last question: Since esophageal carcinoma is unique in its ability at submucosal infiltration proximal to the cancer, have the authors examined biopsy specimens around the cancer itself to see if there is any kind of field effect in terms of the p53 or c-*erb* B-2 expression?

I wish to thank the Association for the privilege of the floor.

DR. HAROLD J. WANEBO (Providence, Rhode Island): Dr. Jurkiewicz, Members, and Guests. This is certainly a very provocative paper, and I rise to ask two questions.

In the initial presentation in the *New England Journal of Medicine,* as I understand it, it was the positive expression of the c-*erb* B-2, that is, the overexpression of this oncogene which correlated with poor outcome. And it was the suggestion that this might be of value perhaps in selecting patients for treatment.

In this case, it's the negative expression that seems to correlate with a better outcome. And I guess that it must imply that those patients are more sensitive to chemotherapy, which is of interest itself, and that may be a point they are making. I suppose if one had a group of esophageal cancer patients who were not treated with chemotherapy, would just the opposite prevail? That is, would those that are erb positive actually do worse? I'd like to have an explanation of that.

The second question that I was going to pose was also asked: have you had a chance to look at the margins on these patients in the resected specimen to see if the same expression exists? In head and neck cancer, there is some very interesting data from Hopkins that suggests that in histologically negative margins, the presence of p53 expression is correlated with a high local recurrence rate. Although you have very good results—I think it's in the 60% range in those who are sterilized, with chemotherapy—it leaves 40% who recur.

In the patients who fail, it would be of interest to know whether they have expression in their histologically negative tissue? It would require going back and looking at your resected specimens, but may turn out to be an important tissue marker for recurrence.

This is a very interesting paper. Thank you for allowing me to discuss it.

DR. WILLIAM C. WOOD (Atlanta, Georgia): Dr. Jurkiewicz, Dr. Copeland, Members, and Guests. I, too, wish to congratulate the authors on producing data on molecular markers for prognosis in adenocarcinoma of the esophagus, an extremely vexing tumor.

I think there are several possible explanations for the results