Evaluation of Immunoreactivity for erbB-2 Protein as a Marker of Poor Short Term Prognosis in Gastric Cancer

Yutaka Yonemura, Itasu Ninomiya, Akio Yamaguchi, Sachio Fushida, Hironobu Kimura, Shigekazu Ohoyama, Ituo Miyazaki, Yoshio Endou, Motothiro Tanaka, and Takuma Sasaki

ABSTRACT

Using a polyclonal antibody that is monospecific for the erbB-2 onco-
gene product, an immunohistochemical study of the expression of erbB-
2 protein was performed in formalin-fixed paraffin-embedded tissue
sections from 260 primary gastric cancers. erbB-2 protein expression in
which the reaction was localized to the cell membranes was observed in
31 (11.9%) cancers. All nontumor cells and normal gastric epithelium
were negative for membrane staining. There was not a significant asso-
ciation between erbB-2 staining and histological type or venous invasion.
erBB-2 protein expression was associated with serosal invasion, lymph
node metastasis, and lymphatic invasion. In addition, erbB-2 protein
expression correlated with a high number of lymph node metastases.
Furthermore, the risk of recurrence in lymph node was over 3 times
higher in patients with erbB-2 protein-positive tumors than in those with
erbB-2 protein-negative ones. When erbB-2 protein expression and the
clinical parameters were entered simultaneously into the Cox regression
model, erbB-2 protein expression emerged as an independent prognostic
indicator. Patients with erbB-2 protein-positive tumors had 5-fold greater
relative risk of death, as compared with those with erbB-2 protein-
negative tumors. These results indicate that erbB-2 protein expression is
an important independent prognostic indicator in gastric cancer. The high
malignant potential of erbB-2 protein-positive tumors may be associated
with the very high potential for lymph node metastasis.

INTRODUCTION

The erb-B2 gene was first isolated due to its homology with
v-erbB and human epidermal growth factor receptor probes (1). This
gene was mapped on chromosome 17 at q21 (2) and codes
for a putative membrane receptor for which a ligand has not
yet been identified. This oncogene generates a mRNA of 4.8
kilobases (3), the protein product of which is a glycoprotein of
the tyrosine kinase family with a molecular weight of 185,000
(4).

Amplification of the erb-B2 gene has been demonstrated in
adenocarcinomas arising at a number of different sites (5, 6).
Among them, erb-B2 alterations have been intensively investi-
gated in mammary neoplasia, where erb-B2 gene amplification
was associated with high level overexpression of normal size
mRNA and protein products (7, 8). Recent studies have shown that
the erb-B2 proto-oncogene is amplified in 25--33% of
human mammary carcinomas and that there is a significant
association between erb-B2 amplification and prognosis, as well
as with lymph node metastasis (7, 9, 10).

A number of factors that are useful for the prediction of the
course of gastric cancer have been identified (11, 12). One of
the most important factors affecting prognosis for patients with
gastric cancer is lymph node metastasis (11--13). However, it is
sometimes difficult to assess lymph node metastasis in patients
with gastric cancer, preoperatively or intraoperatively. Al-
though the correlation of erbB-2 protein expression with nodal
status of mammary cancer has been reported in several studies
(14--16), there has been no report describing the interrelation-
ship between erbB-2 protein expression and lymph node metas-
tasis in gastric cancer (17, 18).

Recently, several authors have shown that the membrane
staining of tumor cells by immunohistochemistry, using specific
antibodies against erbB-2 protein, is a reliable marker of erbB-
2 gene amplification (9, 10, 15, 16). Immunohistochemical
evaluation of formalin-fixed paraffin-embedded tissue is sim-
pler and has a greater potential for widespread application than
DNA analysis by Southern blotting. We analyzed 260 gastric
cancers immunohistologically, using the antisera specific for
the erbB-2 protein, and the erbB-2 protein expression data were
correlated with clinicopathological data and prognosis. We report here that there is a significant association between erbB-
2 protein expression and lymph node involvement.

MATERIALS AND METHODS

Patients and Tissue Samples. Two hundred and sixty patients with
primary gastric cancer, who were diagnosed and treated at the Depart-
ment of Surgery II, Kanazawa University, between 1980 and 1989,
were entered in this study. Tumor specimens were fixed in 10% formalin
and embedded in paraffin. All the patients underwent total or subtotal
gastrectomy combined with extensive lymph node dissection. Sixty-
six patients with early gastric cancers, in which the tumor invasion
was confined to the mucosa or submucosa, were treated only by surgical
procedures, and 197 patients having advanced gastric cancers were
additionally treated with cytotoxic drugs. Two regimens were used,
consisting of type A, mitomycin C (10 mg x 2) plus tegafu (600 mg/
2 years), and type B, tegafu (600 mg/day for 2 years). Types A
and B were used in 92 cases and 105 cases, respectively. Patients who
died of causes other than recurrence of gastric cancer were not included
in the study.

Immunohistochemical Study of erbB-2 Protein Expression. Sections
from the primary tumor of each patient were stained with polyclonal
antibody against erbB-2 protein (pAb-1 (Triton Bioscience Inc., Ala-
meda, CA)). pAb-1, a polyclonal anti-pi85 antiserum, was prepared by
immunizing rabbits with a synthetic peptide corresponding to amino
acid residues 886--880, which comprise the tyrosine kinase domain of
the predicted human erb-B2 (19). Immunoblotting analysis showed that
this antibody specifically reacted with the protein of M, 185,000. After
the 4-μm sections underwent dewaxing and endogenous peroxidase
activity was blocked by incubation for 20 min in 1% hydrogen peroxide
in methanol, the slides were preincubated with 10% normal goat serum
in PBS2 for 15 min, to diminish nonspecific binding of the second
antibody. The slides were then incubated overnight at 4°C with poly-
clonal antibody. The dilution used was 1:20. After washing with PBS,
the slides were incubated with biotinylated goat anti-rabbit immuno-
globulin (DAKO Patts, Copenhagen, Denmark) at a dilution of 1:20
and B were used in 92 cases and 105 cases, respectively. Patients who
died of causes other than recurrence of gastric cancer were not included
in the study.

Received 7/2/90; accepted 11/12/90.

The costs of publication of this article were defrayed in part by the payment
of page charges. This article must therefore be hereby marked advertisement
in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Surgery II, School of
Medicine, Kanazawa University, Takara-Machi 13-1, Kanazawa City, Ishikawa-
Ken, Japan.

2 The abbreviations used are: PBS, phosphate-buffered saline; EGFR, epider-
mal growth factor receptor.
Finally, the slides were stained with 0.3% methyl green, dehydrated, and mounted.

An antiserum absorbed by the peptide (1 mg/ml) was used as the control. Furthermore, each run included a positive control slide, which had previously proved to be strongly positive for the erbB-2 protein. Only tumors that were definitely membrane positive were considered positive for the overexpression of the erbB-2 protein (9, 15).

To study the cross-reactivity of this antibody (pAb-1) with EGFR, pAb-1 and a monoclonal antibody against EGFR (Transformation Research Inc., Framingham, MA) (20) were reacted with each of the two slides of the same specimens from 97 tumors, and the staining patterns of these two antibodies were compared with each other. In addition, paraffin-embedded A431 cells, which are known to have amplified EGFR gene and overexpression of EGFR, were stained using these two antibodies. It has been confirmed that these two antibodies can react with the antigens in both frozen and paraffin-embedded sections (19, 20).

Statistical Analyses. Statistical analyses of data were performed by the \( \chi^2 \) test. The outcomes of different groups of patients were compared by the generalized Wilcoxon test. The Cox proportional hazard model (21) was used in multivariate regression analyses of survival data. Throughout the present report, the information from a previous publication is used for the description and classification of the variables (22).

RESULTS

There were 31 (11.9%) cancers with evidence of erbB-2 protein expression in which the reaction was localized to the cell membrane (Fig. 1, A and B). Only 8 of 31 erbB-2-positive tumors showed homogeneous staining of all tumor cells in the sections. In contrast, the other 23 tumors had focal areas of intense erbB-2 staining, and these tumors showed heterogeneous expression of erbB-2 protein (Fig. 1C).

With regard to the experimental results on the cross-reactivity of pAb-1 with EGFR, 8 of 97 tumors showed positive staining for erbB-2 and EGFR. Neither erbB-2 nor EGFR was stained in 49 tumors, and either erbB-2 or EGFR was stained in 40 tumors. In addition, positive staining for EGFR in A431 cells was observed using a monoclonal antibody against EGFR, but we could not find any staining in A431 cells by pAb-1. These studies indicate that pAb-1 is detecting erbB-2 product without cross-reactivity with EGFR. Positive staining was seen in malignant epithelium only, and no significant staining was seen in normal or metaplastic adjacent mucosa (Fig. 1D). The correlation of erbB-2 protein expression and clinicopathological parameters is shown in Table 1. There was essentially no correlation of erbB-2 protein expression and histological type or venous invasion. Regarding the histological type, 16% (17 of 106) of differentiated adenocarcinomas and 11% (14 of 123) of poorly differentiated and signet ring cell carcinomas were positively stained for erbB-2 protein. However, the presence of erbB-2 protein was correlated with size of primary tumor, serosal invasion, and nodal status. In addition, a strong relationship between erbB-2 protein expression and lymphatic invasion was observed (\( P = 0.001 \)). When analysis was performed...
with ≥7 involved nodes revealed erbB-2 protein expression. There was a significant increase in incidence of erbB-2 protein expression in patients with ≥7 lymph nodes involved with disease.

In addition, 3 of 63 early gastric cancers showed erbB-2 expression, and 2 (67%) of these 3 tumors had lymph node metastases. On the other hand, only 7 (11%) of 60 erbB-2-negative tumors had nodal involvement. The frequency of lymph node metastases between these two groups was statistically significant.

During the follow-up period of 6 months to 10 years, only 92 (40%) of 229 patients with erbB-2 protein-negative tumors died of this disease, compared with 22 (71%) of 31 patients with erbB-2 protein-positive tumors. Patients with erbB-2 protein-positive tumors died significantly earlier than those with erbB-2 protein-negative tumors. However, there was no significant difference in survival between the early cancer patients with erbB-2-positive, and -negative tumors. On the other hand, 28 of 197 advanced gastric cancers were positively stained for erbB-2 protein. The advanced cancer patients with erbB-2 protein-positive tumors had poorer prognoses than did the patients with erbB-2-negative tumors (P < 0.05).

For association between erbB-2 protein expression and number of positive lymph nodes, a strong trend was noted. This analysis showed that 4 of 91 (4.3%) of patients with no involved nodes, 5 of 62 (8.1%) with 1 to 6 involved nodes, and 20 of 92 (21.7%) with ≥7 involved nodes revealed erbB-2 protein expression. There was a significant increase in incidence of erbB-2 protein expression in patients with ≥7 lymph nodes involved with disease.

In addition, 3 of 63 early gastric cancers showed erbB-2 expression, and 2 (67%) of these 3 tumors had lymph node metastases. On the other hand, only 7 (11%) of 60 erbB-2-negative tumors had nodal involvement. The frequency of lymph node metastases between these two groups was statistically significant.

During the follow-up period of 6 months to 10 years, only 92 (40%) of 229 patients with erbB-2 protein-negative tumors died of this disease, compared with 22 (71%) of 31 patients with erbB-2 protein-positive tumors. Patients with erbB-2 protein-positive tumors died significantly earlier than those with erbB-2 protein-negative tumors. However, there was no significant difference in survival between the early cancer patients with erbB-2-positive, and -negative tumors. On the other hand, 28 of 197 advanced gastric cancers were positively stained for erbB-2 protein. The advanced cancer patients with erbB-2 protein-positive tumors had poorer prognoses than did the patients with erbB-2-negative tumors (Fig. 2). There was no difference in survival between the patients who received type A or type B chemotherapeutic regimens. When a follow-up study was made on 167 node-positive patients, patients with erbB-2 protein-negative tumors survived significantly longer than those with erbB-2 protein-positive tumors (Fig. 3).

To compare the prognostic significance of erbB-2 staining, histological type, tumor size, serosal invasion, nodal status, hepatic metastasis, and peritoneal metastasis, multivariate analyses were performed (Table 2). Significant variables for overall survival were serosal invasion, nodal status, hepatic metastasis, and peritoneal metastasis, and erbB-2 protein tissue status. However, histological type and tumor size were not significant. In addition, the erbB-2 tissue status and other clinical parameters of patients with advanced gastric cancer were analyzed by a Cox proportional hazard model. As a result, erbB-2 tissue status was also judged as an independent prognostic factor (Table 3).
been shown to be amplified in gastric cancer, although the exact role of various proto-oncogenes in the pathogenesis of gastric cancer remains unclear. Several proto-oncogenes have been shown to have malignant potential of various tumors (25-27). Recently, the interrelationship between erbB-2 protein expression and biological behavior is not clear. Additionally, the interrelationship between their expression and biological behavior is not clear.

Recently, the erbB-2 gene has been reported to be often amplified in adenocarcinomas of the breast (7-10), stomach (17), and colon (6). Immunohistochemically, 14 to 17% of breast cancers have been reported to be positively stained for erbB-2 protein (9, 10, 14). Amplification of the erbB-2 proto-oncogene, detected by Southern blot analysis, in gastric cancer has been reported to be about 8% (17). This value is slightly lower than our result (12%). However, there has been no report analyzing the relationship of DNA amplification and protein expression of the erbB-2 oncogene in gastric cancer. In breast cancers, Berger et al. (10) reported that most of the tumors which contained amplified gene copies also contained detectable levels of erbB-2 protein but that some tumors with no apparent erbB-2 gene amplification also showed immunohistochemically demonstrable levels of erbB-2 protein. In our study, only 8 of 31 erbB-2-positive tumors showed homogeneous staining of all tumor cells in the sections. On the other hand, 23 of 31 tumors had focal areas of intense erbB-2 staining. The findings suggested that these tumors might be heterogeneous and that amplified erbB-2 gene copies could sometimes go undetected by Southern blot analyses. In other studies, both uniform (15, 29), and patchy (30, 31) staining of positive cases has been found. This may be partly due to variation in fixation or antibodies used. Thus, small numbers of tumor cells that have elevated levels of the erbB-2 protein can be detected by staining which would not be observed with the other methods. In this sense, immunohistochemical evaluation of formalin-fixed paraffin-embedded tissue using pAB-1 has a greater potential for retrospective analysis of the prognostic significance than DNA analysis by Southern blotting.

Regarding the interrelationship between histological type and erbB-2 protein expression in gastric cancer, Falck and Gullick (18) reported that prominent staining was restricted to well differentiated adenocarcinomas. In our study, the positive rate of expression of erbB-2 protein in the differentiated adenocarcinomas was higher than that in the poorly differentiated adenocarcinomas. However, there was no statistical difference between these two groups. Falck and Gullick (18) reported that 6 (10%) of 61 poorly differentiated adenocarcinomas showed erbB-2 staining. In early cancers of our series, the positive rate of erbB-2 protein expression of differentiated adenocarcinomas was only 6%; the reason for the difference between our result and others' (17, 18) may have derived from the numbers of early cancers examined. The fact that positive staining was also found in poorly differentiated and signet ring cell carcinomas indicates that erbB-2 is not uniquely linked to a specific differentiated type.

In studies of breast cancer, amplification of the erbB-2 gene has been shown to be associated with poorer prognosis for the patients (7-9). However, there have been no reports describing the interrelationship between erbB-2 protein expression and malignancy of gastric cancer. The present report demonstrated that erbB-2 protein expression, as well as conventional prognostic factors, is a powerful independent prognostic indicator of gastric cancer. Patients with erbB-2 protein-positive tumors had 5-fold greater relative risk of death than those with erbB-2 protein-negative tumor. Our results also showed that erbB-2 protein expression was associated with wall invasion, lymph node metastasis, and lymphatic invasion. In addition, erbB-2 protein expression correlated with a high number of lymph node metastases. Furthermore, the risk of recurrence in lymph node was over 3 times higher in patients with erbB-2 protein-
positive tumors than in those with erbB-2 protein-negative tumors. These results are the same as were reported in breast cancer (7). The current results indicate that the high malignant potential of tumors with erbB-2 protein expression may be closely associated with the potential for lymph node metastasis.

The erbB-2 protein is similar in structure to epithelial growth factor receptor and is presumed to be a membrane receptor, but the ligand(s) remains unknown. It seems that binding of unknown ligand(s) to erbB-2 protein might lead, via increased protein kinase activity, to the promotion of cell replication. We previously reported that EGFR-positive tumors had a poorer prognosis than did EGFR-negative tumors and that the growth fractions of tumors expressing EGFR were significantly higher than those of EGFR-negative tumors (26). Slamon et al. (7) speculated that a gene encoding a putative growth factor receptor, when expressed in inappropriate amounts, may give a growth advantage to the cells expressing it. We are now studying the correlation of erbB-2 protein expression with the growth fraction of tumors.

In conclusion, our results indicate that erbB-2 protein expression is an important independent prognostic indicator in gastric cancer. The high malignant potential of erbB-2 protein-positive tumors may be associated with the high metastatic potential for the lymph nodes. When the biopsied materials of gastric cancer are stained by this antibody, the erbB-2 tissue status of each of the tumors can be diagnosed preoperatively.

As others have noted, the recent demonstration that a monoclonal antibody to this oncoprotein could inhibit the growth of human breast carcinoma cells in vitro (26). Slamon et al. (7) speculated that a gene encoding a putative growth factor receptor, when expressed in inappropriate amounts, may give a growth advantage to the cells expressing it. We are now studying the correlation of erbB-2 protein expression with the growth fraction of tumors.

In conclusion, our results indicate that erbB-2 protein expression is an important independent prognostic indicator in gastric cancer. The high malignant potential of erbB-2 protein-positive tumors may be associated with the high metastatic potential for the lymph nodes. When the biopsied materials of gastric cancer are stained by this antibody, the erbB-2 tissue status of each of the tumors can be diagnosed preoperatively.

As others have noted, the recent demonstration that a monoclonal antibody to this oncoprotein could inhibit the growth of human breast carcinoma cells in vitro (26). Slamon et al. (7) speculated that a gene encoding a putative growth factor receptor, when expressed in inappropriate amounts, may give a growth advantage to the cells expressing it. We are now studying the correlation of erbB-2 protein expression with the growth fraction of tumors.

In conclusion, our results indicate that erbB-2 protein expression is an important independent prognostic indicator in gastric cancer. The high malignant potential of erbB-2 protein-positive tumors may be associated with the high metastatic potential for the lymph nodes. When the biopsied materials of gastric cancer are stained by this antibody, the erbB-2 tissue status of each of the tumors can be diagnosed preoperatively.

As others have noted, the recent demonstration that a monoclonal antibody to this oncoprotein could inhibit the growth of human breast carcinoma cells in vitro (26). Slamon et al. (7) speculated that a gene encoding a putative growth factor receptor, when expressed in inappropriate amounts, may give a growth advantage to the cells expressing it. We are now studying the correlation of erbB-2 protein expression with the growth fraction of tumors.

In conclusion, our results indicate that erbB-2 protein expression is an important independent prognostic indicator in gastric cancer. The high malignant potential of erbB-2 protein-positive tumors may be associated with the high metastatic potential for the lymph nodes. When the biopsied materials of gastric cancer are stained by this antibody, the erbB-2 tissue status of each of the tumors can be diagnosed preoperatively.

As others have noted, the recent demonstration that a monoclonal antibody to this oncoprotein could inhibit the growth of human breast carcinoma cells in vitro (26). Slamon et al. (7) speculated that a gene encoding a putative growth factor receptor, when expressed in inappropriate amounts, may give a growth advantage to the cells expressing it. We are now studying the correlation of erbB-2 protein expression with the growth fraction of tumors.

In conclusion, our results indicate that erbB-2 protein expression is an important independent prognostic indicator in gastric cancer. The high malignant potential of erbB-2 protein-positive tumors may be associated with the high metastatic potential for the lymph nodes. When the biopsied materials of gastric cancer are stained by this antibody, the erbB-2 tissue status of each of the tumors can be diagnosed preoperatively.

As others have noted, the recent demonstration that a monoclonal antibody to this oncoprotein could inhibit the growth of human breast carcinoma cells in vitro (26). Slamon et al. (7) speculated that a gene encoding a putative growth factor receptor, when expressed in inappropriate amounts, may give a growth advantage to the cells expressing it. We are now studying the correlation of erbB-2 protein expression with the growth fraction of tumors.
Evaluation of Immunoreactivity for erbB-2 Protein as a Marker of Poor Short Term Prognosis in Gastric Cancer

Yutaka Yonemura, Itasu Ninomiya, Akio Yamaguchi, et al.