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Expression of *Her2/neu* oncogene product p185 in correlation to clinicopathological and prognostic factors of gastric carcinoma *,**

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Summary. The expression of the *Her2/neu* gene product p185 was retrospectively analyzed in 58 patients with gastric carcinoma. The results were correlated to various clinicopathological and prognostic factors. Positive membrane staining for p185 could be detected in 38% of the patients (22/58). Membrane staining was significantly greater in well and moderately differentiated tumors of the intestinal type when compared with poorly differentiated lesions and carcinomas of the diffuse type ($P < 0.01$). Positive membrane staining did not correlate with site and tumor stage, but T1 lesions had less membrane staining than more advanced primary tumors. Overall survival showed no difference between p185-positive and negative cases. Multivariate analysis defined a subgroup of curatively resected patients with stage III and IV disease that had a statistically significant poorer survival when p185 was overexpressed ($P = 0.005$). Overexpression of the *Her2/neu* product p185 appears to be associated with intestinal-type gastric carcinoma and may help in identifying a subset of patients at increased risk for shorter survival.

Key words: *Her2/neu* oncogene product p185 – Gastric carcinoma – Clinicopathological variables – Prognosis

Introduction

Alterations of protooncogenes either in gene structure or gene copy presumably play an etiological role in carcinogenesis. One such gene with great potential for malignant transformation is the *Her2/neu* gene located on chromosome 17q21 (Fukushige et al. 1986; Di Fiore et al. 1987). This gene generates a messenger RNA of 4.8×10^3 bases (kb), which encodes a transmembrane glycoprotein (p185) with a molecular mass of 185 kDa (Schechter et al. 1984; Akiyama et al. 1986). p185 is similar, but distinct from epidermal growth factor receptor (Yamamoto et al. 1986), and its proposed ligand is a 30-kDa factor (Lupu et al. 1990).

Amplification of the *Her2/neu* gene has been demonstrated in a variety of adenocarcinomas, such as those in the breast and colon (Yokota et al. 1986; Tal et al. 1988; Gutman et al. 1989). It has been shown in breast carcinoma that amplification of this gene is strongly correlated with survival (Slamon et al. 1987; Slamon et al. 1989a). In gastric carcinoma *Her2/neu* was found to be amplified in 10% of the tumors (Houldsworth et al. 1990). Since amplification of the *Her2/neu* gene results in elevated levels of *Her2/neu* mRNA and its protein product (Slamon et al. 1989a), immunohistochemical studies using antibodies against the oncogene product subsequently showed overexpression in 19% of gastric adenocarcinoma (Falck and Gullick 1989). In contrast to breast carcinoma, however, there are only a few reports about the prognostic significance of the *Her2/neu* protein product, indicating that it may well be of prognostic relevance (Yonemura et al. 1991a, b).

In the present study we therefore analyzed alterations of the *Her2/neu* protein product in gastric carcinoma and correlated the results with different clinicopathological data and with the prognosis of the patients.

Materials and methods

Patients and tissue samples. A group of 58 patients with resectable primary adenocarcinoma of the stomach was retrospectively analyzed. The tumors to be studied by immunohistochemistry were randomly selected on the basis of tissue availability and excellent tissue preservation defined as non-necrotic cancerous lesion. The median age of the patients was 66.5 years (range: 25–83 years), 36 were male and 22 were female (male : female ratio = 1 : 0.6). All patients were

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Abbreviations: PBS, phosphate-buffered saline; BSA, bovine serum albumin

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operated upon at the Department of Surgery at Memorial Sloan Kettering Cancer Center (MSKCC) between January 1985 and July 1990. The resection of the primary tumor included systematic lymphadenectomy in compartments I and II (UICC 1987) when there was potential for a curative resection. In 46 patients the resection was considered to be potentially curative (R0-resection), while in 12 patients a palliative resection was performed (R1: $n=4$; R2: $n=8$). The site of the primary lesion was the proximal third of the stomach including the gastroesophageal junction in 21 patients, the distal third (antrum/pylorus) in 24 and the body in 9 patients. Four patients showed involvement of the whole stomach.

Immunohistochemistry. Prior to immunohistochemistry all slides were reviewed (C.U.) for exact grading (Oota and Sobin 1977) and staging (UICC 1987) of the tumors, which were classified according to Laurén (1965). Two of the most representative sections of the tumor and, for comparison, normal gastric mucosa were chosen for this study.

The avidin-biotin complex/immunoperoxidase technique was used for immunohistochemistry (Hsu et al. 1981). Formalin-fixed and paraffin-embedded tissue sections (5 μm) were deparaffinized and treated in 1% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min to block endogenous peroxidase activity. After three washes in distilled water the sections were treated in 0.05% saponin (Sigma Chemical Co., St. Louis, Mo., USA) in distilled water for 30 min for enzymatic digestion. Tissue sections were incubated with 10% normal goat serum (Cappel, West Chester, Pa., USA) in 2% bovine serum albumin (BSA) and PBS for 30 min. Blocking serum was aspirated and a rabbit polyclonal antibody to the p185 *Her2/neu* protein product (antiserum no. 60, dilution 1:1500 in 2% PBS/BSA; gift of D. Slamon, University of California, Los Angeles, USA) was applied. The specificity of this antibody and its consistency with the results of Southern and Northern blotting were confirmed in earlier studies (Slamon et al. 1989b). Sections were incubated with the primary antibody overnight at 4° C. After three washes with PBS, biotinylated anti-rabbit IgG (dilution 1:1000 in PBS; Vector Laboratories, Burlingame, Calif., USA) was used as secondary antibody for 30 min. The sections were then washed again with PBS and incubated with the avidin-biotin complex (dilution 1:25 in PBS, 30 min; Vector Laboratories). Following treatment with 3′,3′-diaminobenzidine (Sigma Chemical Co.) as chromogen, the sections were washed with distilled water, counterstained with hematoxylin and mounted with Permount. In negative controls the primary antibody was substituted by 2% PBS/BSA. Positive controls were sections of a breast carcinoma known to show overexpression of p185.

The sites of immunoprecipitation were evaluated by light microscopy. Only cell membrane staining was considered to be positive for p185. The staining intensity was classified from 1+ (light staining) to 3+ (dark staining, and equal to the control slide). Positive reactions were also measured in percentages and placed into four rough categories as suggested in other studies (Pavelic et al. 1990): 0%, 1%–30% (weak), 31%–60% (moderate) and 61%–100% (strong) staining.

Variables and statistical analysis. The staining pattern of p185 was analyzed in terms of sex and age of the patients, histological grade and type of the tumor, stage and site, and whether the resection was curative or palliative. Statistical significance of differences between the various variables was determined by Fisher's exact *t*-test.

Since there was no immediate postoperative mortality and death in all patients resulted from recurrent disease, all patients were included in the survival analysis. Survival data were calculated according to Kaplan-Meier. Prognostic factors were examined univariately by the Wilcoxon and log-rank tests for their relationship to survival independently of the staining. Since some of these factors were interrelated, the Cox proportional-hazard model for multivariate analysis was used to determine the extent to which each factor contributed independently to prediction of survival. The prognostic significance of *Her2/neu* protein product staining was then analyzed overall and within prognostic groups.

Results

Staining pattern of p185 and clinicopathological factors

Positive cell membrane staining was only seen in carcinomas, but not in normal gastric epithelium. We observed overexpression of the *Her2/neu* product on the basis of membrane staining in 38% of the tumors analyzed (22 of 58 cases; Fig. 1). The staining pattern was predominantly detected at the lateral and basal membrane sites with

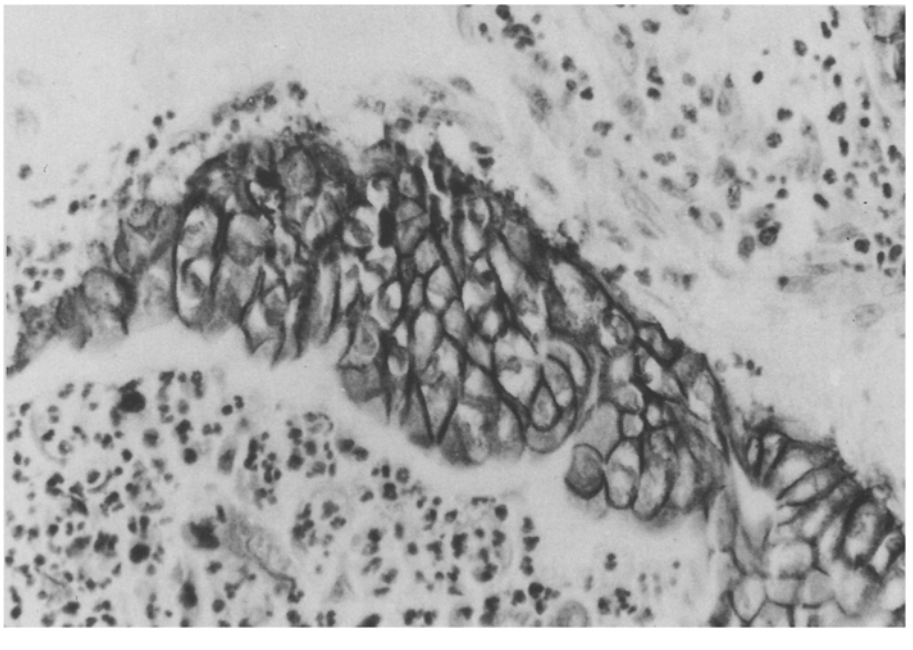

Fig. 1. Well-differentiated gastric adenocarcinoma, intestinal type, showing intense p185 membrane and weak cytoplasmic staining. (Magnification: ×400)

weak or no staining at the luminal site. Among the positive cases almost half (45.5%) had strong membrane expression (>60%–100% of the cells) of p185, 4 lesions showed moderate (>30%–60%) and 8 tumors had weak expression (<30%). Granular cytoplasmic staining was seen in 59% of the carcinomas (34/58).

The majority of the positive cases were moderately or well differentiated (Fig. 1). Combined, they had a 56.3% positive staining rate, while only 15.4% of the poorly differentiated carcinomas showed immunoreactivity at the membranes ($P=0.002$). In terms of the histological type, intestinal and mixed but not diffuse carcinomas showed expression of *Her2/neu* protein product ($P<0.001$; Table 1).

Membrane staining was not influenced by tumor stage or nodal status. Regarding the degree of membrane staining, however, more invasive tumors had a greater percentage of positive staining than early gastric carcinomas (Table 2).

The site of the tumor did not have a statistically significant influence on the overall immunoreactivity of the *Her2/neu* product. However, only 1 of 21 proximal lesions, but 6 of 37 carcinomas at other sites of the stomach showed strong overexpression of p185 in almost all cells (>60%). Of those, 5 cases were located at the antrum.

Additionally, positive membrane staining was not influenced by the sex or age of the patient and whether the resection was curative or palliative.

Survival

All patients were followed for a mean of 1.5 years. Although this follow-up period is relatively short, survival analysis seems to be justified, since 50%–70% of all patients with gastric carcinoma die within 2 years even after potentially curative resections (Ovaska et al. 1989). The overall 3-year survival was 24% (median = 1.4 years). None of the 7 patients alive after 3 years had yet died at the time of this analysis. In our study the histological grade and type did not influence survival. The log-rank test for comparison of groups was used. Depth of tumor infiltration ($P=0.002$), nodal status ($P=0.008$), the presence of distant metastases ($P=0.006$), tumor stage ($P=0.0002$) and whether the resection was curative or palliative ($P<0.0001$) were the most predictive prognostic factors for survival. The presence of positive immunostaining, however, was not related to survival for the study population as a whole (Fig. 2).

In the multivariate analysis, tumor stage as a linear predictor was the major independent prognostic factor. Among other factors tested (e.g. histological type) only the quality of the resection (curative or palliative) had additional prognostic significance when included in the Cox regression model together with stage (Table 3). Considering these factors patients were grouped into good (stage I and II, curative resection), intermediate (stage III and IV, curative resection) and poor risk (stage III and IV, pallia-

Table 1. Expression of p185 in correlation to histological grade (Oota and Sobin 1977) and type (Laurén 1965)

Histological grade and type	Immunoreactivity of p185		
	Positive (*n*)	Negative (*n*)	Positive (%)
Grade			
Well/moderate ($n=32$)	18	14	56.3
Poor ($n=26$)	4	22	15.4*
Type			
Intestinal ($n=43$)	21	22	48.8
Diffuse ($n=13$)	–	13	0.0**
Mixed ($n=2$)	1	1	50.0

* $P=0.002$, ** $P<0.001$

Table 2. Expression of p185 and depth of tumor infiltration (T stage)

T stage	Immunoreactivity of p185[a]		
	Positive<30%	Positive>30%	Total no. positive
T1 ($n=7$)	3	–	3
T2 ($n=17$)	3	4	7
T3 ($n=31$)	2	9	11
T4 ($n=3$)	–	1	1
Total ($n=58$)	8	14	22

[a] Rank correlation between T stage and degree of staining among positive cases ($n=22$; $P<0.001$)

Table 3. Results of multivariate analysis

Variable	*P*	Hazard ratio
Tumor stage	0.0007	2.32[a]
Quality of resection (curative/palliative)	0.0281	2.76

[a] Assuming a constant proportional increase in hazard ratio between successive stages

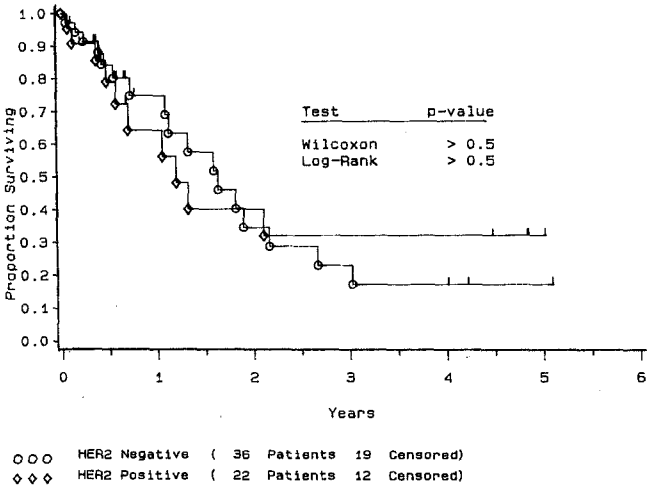

Fig. 2. Overall survival with respect to *Her2/neu* immunoreactivity ($n=58$; Kaplan-Meier); *tick marks* indicate last follow-up

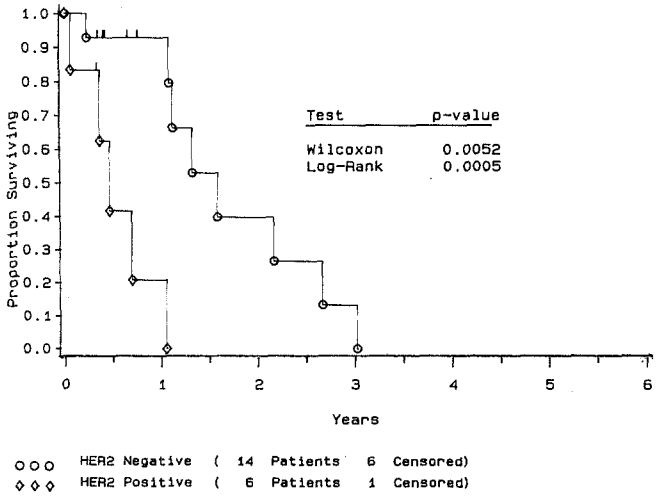

Fig. 3. Survival of curatively resected stage III and IV gastric adenocarcinoma with respect to *Her2/neu* immunoreactivity ($n=20$; Kaplan-Meier); *tick marks* indicate last follow-up

tive resection). Positive membrane staining was significantly related to survival only in the intermediate-risk group (Fig. 3). It is noteworthy that the numbers for the histology and site of the tumor were equally distributed among the patients of this group.

Discussion

Despite possible improvements in chemotherapy, surgical resection still represents the only hope of cure for patients with gastric carcinoma (Shiu et al. 1987; Wilke et al. 1990). Even after potentially curative resections, however, the prognosis of gastric carcinoma in the western part of the world remains poor (Ovaska et al. 1989; Lundegardh et al. 1986) and depends on tumor stage, the number of metastatic lymph nodes (Ovaska et al. 1989; Jaehne et al. 1992) and perhaps DNA ploidy (Nanus et al. 1989).

Particularly with respect to the ploidy status of malignancies, alterations on the genetic level are thought to be involved in carcinogenesis. This could be well demonstrated in colorectal carcinoma (Fearon and Vogelstein 1990). Additionally, specific genetic defects may be of prognostic significance. It has been shown that amplification of the *Her2/neu* gene plays an important prognostic role in breast carcinoma (Slamon et al. 1987). In gastric carcinoma the *Her2/neu* gene has been found to be amplified in 10% of the carcinomas (Houldsworth et al. 1990) and amplification is frequently associated with aneuploidy (Oda et al. 1990). There are, however, only a few reports about the biological relevance and prognostic significance of the *Her2/neu* oncogene in gastric carcinoma. Japanese authors claimed that patients with overexpression of the gene product have a poorer survival (Yonemura et al. 1991 a, b). In order to gain further insight into the role of the *Her2/neu* gene in gastric carcinoma, we therefore evaluated alterations of the gene product p185 with respect to various histological and prognostic variables.

We found a positive immunoreactivity for p185 in 38% of the cases studied. The percentage of tumors with positive immunostaining is higher than that reported in other series (Falck and Gullick 1989; Yonemura et al. 1991 a, b; Kameda et al. 1990) and when compared with the incidence of *Her2/neu* amplified cases (Yokota et al. 1986, 1988). It may be due to the high specifity of the antibody (Slamon et al. 1989 a) and/or to variations in the method employed, mainly fixation, since it has been demonstrated that some antibodies, including the one used here, show different staining results depending on the kind of fixation (Slamon et al. 1989 b). It could be demonstrated that the polyclonal antibody applied in this study gave different staining results when frozen sections were compared with paraffin-embedded tissue. The immunoreactivity was generally less in paraffin-embedded sections. This was relevant, however, only in tumors showing a moderate to weak expression in frozen tissue (Slamon et al. 1989 b). Our staining results indicate that the antibody also shows good immunoreactivity at the basal membranes in paraffin-embedded tissue of gastric cancer; almost 50% of the tumors had a strong positivity for p185. Our finding of cytoplasmic staining remains unclear, but it has also been described by others (Kameda et al. 1990). Since proteins are synthesized in the ribosomes, one may postulate that the antibody detects cytoplasmic precursors of the final product. Studies using electron microscopy will be necessary to localize the cytoplasmic staining specifically.

The difference between the reported numbers of amplified cases on the one hand and positively stained tumors in this study on the other hand may be explained by the sensitivity of Southern blotting. Since some lesions showed heterogeneous staining for p185 one might assume that only a few cells have an amplified *Her2/neu* gene. If this number is small, Southern blotting may be incapable of detecting gene amplification because of a preponderence of obscuring normal cells. Alternatively, a proportion of cells may overexpress *Her2/neu* by other mechanisms not involving gene amplification.

Overexpression of the *Her2/neu* gene product at the basal membrane site was almost exclusively found in well and moderately differentiated carcinomas of the intestinal type. There was a statistically significant difference when compared with results in poorly differentiated lesions and carcinomas of the diffuse type. This result supports earlier findings (Falck and Gullick 1989), but is in contrast to recently published reports (Yonemura et al. 1991 a, b). Differences in the antibodies used for p185 specificity may partly explain this discrepancy (van de Vijer et al. 1988; Berger et al. 1988). The results of immunohistochemistry, however, indicate that overexpression of the *Her2/neu* gene product represents a specific feature of intestinal-type gastric carcinoma.

In contrast to breast carcinoma and other reports on gastric carcinomas, we were unable to find a significant difference between *Her2/neu* amplification and p185-positive and negative cases in terms of tumor stage (Slamon et al. 1987; Yonemura et al. 1991 a, b). As reported by others (Yonemura et al. 1991 b), however, early gastric carcinomas tended to show less membrane staining than

advanced lesions. Concerning the site of the tumors it is noteworth that only one gastroesophageal tumor, but five distal carcinomas showed overexpression of p185 in almost all cells. This difference did not reach statistical significance, which may be due to the relatively small sample size. However, the finding correlates well with the fact that *Her2/neu* gene amplification occurs only rarely in gastroesophageal lesions (A. Albino, MSKCC, personal communication). Biologically, distal lesions are considered to be more favorable than proximal tumors (Shiu et al. 1987; Nanus et al. 1989). Therefore, it remains unclear why distal carcinomas had a higher immunopositivity of the oncogene product, which appears to be a marker for poor prognosis (Slamon et al. 1987). On the other hand, recent studies failed to demonstrate that the site of the primary lesion is of prognostic significance (Jaehne et al. 1992).

Although follow-up is relatively short, our study confirms that survival in patients with gastric carcinoma strongly depends on tumor stage and whether the resection was curative or palliative, but it is independent of the histological grade and type. Earlier studies reported the intestinal type to be biologically and prognostically more favorable (Laurén 1965), but several recent reports indicate that the histological grade and type have no influence on survival (Yonemura et al. 1991 a; Jaehne et al. 1992; Nanus et al. 1989). With respect to the *Her2/neu* product tissue immunoreactivity there was no statistically significant difference between the positive and negative cases, a finding that is different from other data in breast and gastric carcinoma (Slamon et al. 1987; Yonemura et al. 1991 a, b). However, the reports on breast carcinoma could not be entirely confirmed by follow-up studies (Clark and McGuire 1991), and in the two studies of gastric carcinoma from Japan (Yonemura et al. 1991 a, b) there is no information about the number of curative and palliative resections. If this relevant prognostic factor (Shiu et al. 1987; Jaehne et al. 1992) was considered it may well be that *Her2/neu* staining would have been less predictive for survival. On the other hand, our study population may be too small to show an overall prognostic significance of the *Her2/neu* gene product, but our data are supported by an earlier report, which also failed to demonstrate any prognostic significance of p185 (Masood et al. 1991). By multivariate analysis, however, we identified a subset of approximately 1/3 of the patients (stage III/IV and potentially curative resection) among whom positive membrane staining was significantly associated with poorer survival irrespective of the site of the primary lesion.

In conclusion, overexpression of the *Her2/neu* gene product p185 seems to be a specific phenomenon of certain intestinal-type gastric carcinomas. Further studies are necessary to evaluate the relevance of other oncogenes and tumor suppressor genes in the carcinogenesis of gastric carcinoma. Alterations in the expression of the *Her2/neu* product appear not to be of overall prognostic significance, but may help in identifying those patients likely to experience shorter survival when considered together with other major risk factors.

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