GASTROENTEROLOGY 1997;112:1103–1113

The Expression of p185$^{HER-2/neu}$ Correlates With the Stage of Disease and Survival in Colorectal Cancer

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Background & Aims: HER-2/*neu* oncogene encodes a transmembrane tyrosine kinase receptor that is amplified and/or overexpressed predominantly in adenocarcinomas. This phenomenon has been most intensively studied in breast carcinoma where its amplification and overexpression correlate with the overall course of disease and poor prognosis. This study was designed to investigate HER-2/*neu* gene expression in benign and malignant colorectal lesions and to evaluate its prognostic importance in colorectal cancer. **Methods:** Two hundred twenty-one samples of normal colon, benign lesions, and colorectal adenocarcinomas were studied for expression of HER-2/*neu* oncoprotein. Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections of primary tumor and lymph nodes was performed. Immunoprecipitation followed by Western blotting of freshly frozen samples of the same tumors were also performed. **Results:** Normal colon mucosa, benign lesions, and adenocarcinomas clearly differed in the expression levels and histological distribution of p185$^{HER-2/neu}$. Normal mucosa was mostly negative, but significant number of benign lesions and adenocarcinomas overexpressed HER-2/*neu* protein. Adenocarcinomas were significantly more positive than benign lesions. The results show significant correlation with the epithelial abnormality degree and clinical parameters including Dukes' classification and relapse-free and postoperative survival period. **Conclusions:** The p185$^{HER-2/neu}$ rate expression could serve as an independent prognostic factor in patients with p185$^{HER-2/neu}$–positive colorectal malignancies.

Proto-oncogenes, including the HER-2/*neu* gene (also called *c-erbB-2*), represent a family of normal cellular genes involved in cell growth and differentiation. There is much evidence that alterations in the structure of these genes or their amplification or overexpression may play a role in the pathogenesis of some human cancers. The HER-2/*neu* gene encodes a 185-kilodalton transmembrane protein with tyrosine kinase activity that shares approximately 50% of overall homology with epidermal growth factor (EGF) receptor, suggesting a possible role as a membrane receptor.[1,2] This proto-oncogene is frequently expressed at low levels in a variety of human adult epithelial cells[3] and amplified or overexpressed predominantly in carcinomas of glandular epithelial origin and cell lines derived from them.[4–9] This phenomenon has been most intensively studied in breast carcinoma where high levels of HER-2/*neu* expression have been shown to correlate with the overall clinical course of the disease[10] and poor prognosis[11,12] and to predict a poorer response to chemotherapy[13] and endocrine therapy.[14] The expression of HER-2/*neu* protein has also been described as an important independent prognostic indicator in gastric cancer.[15]

Recently, we reported a pilot study on a group of 17 patients with HER-2/*neu*–positive colorectal carcinomas.[16] The report showed a positive correlation between HER-2/*neu* overexpression rate and survival monitored during a 30-month period as well as between overexpression of this oncogene and the time of detection of liver metastases. In the present study, we aimed to determine whether the expression of this protein is related to the progression of disease and whether the intensity of HER-2/*neu* expression can be a useful prognostic marker of colorectal cancer.

Materials and Methods

Patients and Tissue Specimens

This retrospective study involved 221 specimens of normal colon tissue and benign and malignant colon lesions (Table 1). All specimens were obtained through routine sur-

Abbreviations used in this paper: EGF, epidermal growth factor; MAb, monoclonal antibody; SDS, sodium dodecyl sulfate.

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0016-5085/97/$3.00

gery performed at the medical centers of Šibenik and Split, Croatia. The diagnoses were established by standard diagnostic procedures and confirmed histopathologically. Staging was performed by Turnbull's modification of Dukes' staging.[17] The patients were followed up according to a standardized protocol that included laboratory tests (i.e., routine blood count) at 1–2-month intervals, chest radiography, ultrasonography, and computerized tomographic examination of the liver, and endoscopy of the colon at 1-year intervals during the first 3 postoperative years and thereafter in 6–12-month periods. A disease-free interval was defined as the time from resection to the first clinically detectable recurrence of tumor. Causes of death were ascertained from the medical records or autopsy (if performed). Patients who died within 4 weeks of radical surgery were excluded from the analysis. Deaths by other causes were censored observations from the time of death.

None of the patients underwent preoperative radiation or chemotherapy. The study included 128 men and 89 women with age range between 25 and 88 years (mean age, 63.8 years).

Carcinoembryonic antigen estimations were not routinely used and therefore were not analyzed.

Each specimen was routinely fixed in 10% formalin and immersed in melted paraffin. Four micrometer sections were cut and mounted onto glycerine-treated slides. Fresh samples of resected colon carcinoma, immediately adjacent to the segment of tissue that was fixed in formalin, were snap-frozen in liquid nitrogen and stored in a Human Tumor Bank[18] at −80°C until further use. Before inclusion in the study, each specimen was verified by a histopathologist.

Cell Lines

Human breast carcinoma (SK-BR-3) and human colon carcinoma (HT 29 and SW480) cell lines were used. The cells were cultured in Dulbecco's modified Eagle medium (GIBCO, Gaithersburg, MD) supplemented with 10% fetal calf serum. For immunocytochemical studies, the cells were grown in four-chamber slides (Nunc, Roskilde, Denmark) overnight in a humidified atmosphere with 5% CO_2 at 37°C.

Immunohistochemical Detection of Oncogene Proteins

After deparaffinization in xylene, the slides were washed in phosphate-buffered saline (PBS) (three times for 3 minutes). The endogenous peroxidase activity was quenched by 15-minute incubation in methanol with 3% hydrogen peroxide (Sigma Chemical Co., Deisenhofen, Germany). The fixation slides were then cleared with PBS. Nonspecific binding was blocked by applying normal rabbit serum in a humidity chamber in a dilution of 1:10 for 30 minutes. Slides were blotted, and the primary monoclonal antibody (MAb) *c-neu* (Ab-3; Oncogene Science, Uniondale, NY) at a concentration of 5 μg/mL was applied for 15 minutes at room temperature. Slides were washed three times in PBS containing 3%, 2%, and 1% of normal human serum. Secondary antibody (rabbit α mouse; Dako, Glostrup, Denmark), diluted with PBS and normal human serum (40 μL rabbit anti-mouse antibody, 50 μL normal human serum, and 910 μL PBS), was applied for 1 hour at room temperature. Finally, peroxidase–antiperoxidase (Dako) conjugate diluted 1:100 in PBS was applied for 1 hour at room temperature. After washing with PBS, slides were kept in diaminobenzidine tetrahydrochloride for 7 minutes (50 mg in 200 mL PBS with 25 μL of 30% H_2O_2) (DAB; Sigma Chemical Co.), counterstained with hematoxylin for 30 seconds, immersed in saturated lithium carbonate for 30 seconds, and mounted in PBS-glycerol (1:1).

To verify the immunohistochemical results obtained with *c-neu* (Ab-3) antibody, we performed an analysis of some samples with a different mouse MAb (CB11), which was raised against different sequence in the intracellular domain of p185HER-*2/neu*. This antibody was a part of the StrAviGen Super sensitive kit (Bio Genex Laboratories, San Ramon, CA).[19] Complete agreement was found between the two antibodies.

Immunocytochemistry

Cultured cells were washed in PBS after removing the medium and fixed in methanol. Immunocytochemistry was performed according to the procedure described for the tissue sections.

Evaluation of Slides

Each slide was evaluated in the entire tumor area. The concentration of the antigen was assessed by estimating the relative visual intensity of a chromogenic label, and the results were expressed on a 3-point scale as follows: +, weak staining; ++, moderate staining; and +++, strong staining.[20] Each sample was assessed independently by two observers (S.K. and R.S.). There was a 95% initial agreement between them.

Preparation of Tissue for Biochemical Analysis

The frozen tissue samples were pulverized to a fine powder in liquid nitrogen. The powder was redissolved in PBS with 1 mmol/L phenylmethylsulfonyl fluoride and aprotinin (2 μg/mL) as protease inhibitors and subsequently centrifuged at 15,000*g* in an Eppendorf centrifuge (Hamburg, Germany) and ultracentrifuged at 70,000*g*. The supernatant (S1-cytosol) was used in immunoprecipitation and sodium dodecyl sulfate (SDS) electrophoresis.

The pellet (membranes) was placed in lysis buffer (150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 10 mmol/L Tris-HCl, pH 7.2) and ultracentrifuged at 70,000*g*. The supernatant (S2-membrane proteins) was used for immunoprecipitation as well.

The cells were redissolved in lysis buffer with 1 mmol/L phenylmethylsulfonyl fluoride and aprotinin (2 μg/mL) and centrifuged at 15,000*g* in an Eppendorf centrifuge. The supernatant (S3) was used for immunoprecipitation and SDS electrophoresis.

Immunoprecipitation

MAb *c-neu* (3B5, Ab-3; Oncogene Science) (1 μg/mL) was incubated with supernatant (1 mg total proteins of S1,

S2, or S3) for 1 hour on ice with shaking. Immunoprecipitation was followed with the addition of protein A–Sepharose (Pharmacia, Uppsala, Sweden) for the next 2 hours. Reaction was stopped by adding cold lysis buffer and centrifuged in an Eppendorf centrifuge for 5 minutes at 15,000*g*. Immunoprecipitates were washed three more times with cold lysis buffer. Sample buffer for SDS–polyacrylamide gel electrophoresis (PAGE), 50 μL, was added to each precipitate, and the samples were frozen at −20°C.

SDS-PAGE

Electrophoresis was run in a Tris-glycine buffer (0.025 mol/L Tris, 0.19 mol/L glycine, and 1% SDS, pH 8.6) on 7.5% polyacrylamide gel according to the method of Laemmli. Molecular weights were calculated according to the high-molecular-weight standards (GIBCO BRL).

Immunoblotting (Western Blotting)

Electrophoretically separated proteins were electroblotted to nitrocellulose membrane (BA 85, 0.45 μm; Schleicher & Schuell, Dussel, Germany) in electrode buffer (25 mmol/L Tris and 192 mmol/L glycine, pH 8.3) at 150 mA for 45 minutes with a cooling Midget MultiBlot Electrophoretic Transfer Unit (LKB 2051; Pharmacia). Immunodetection of antigen immobilized on nitrocellulose membrane was accomplished by the alkaline phosphatase method. Unoccupied protein binding sites on the nitrocellulose were blocked with 3% bovine serum albumin (Sigma), 0.05% Tween 20 (Sigma), and 5% nonfat milk in PBS for 30 minutes. The membrane was then incubated with primary MAbs *c-neu* (3B5, Ab-3) or P-Tyr (Ab-2) in a concentration of 10 μg/mL in 0.3% bovine serum albumin plus 0.05% Tween 20–PBS for 2 hours at room temperature with agitation. After washing with the same buffer, the membrane was incubated with secondary antibody conjugate in a concentration of 0.2 μg/mL. The reaction was stopped with stop buffer. The membrane was washed in distilled water and dried.

Controls

Control staining was performed either by omitting the primary antibody, by the use of nonimmune serum and irrelevant antibodies, or by preincubating the primary antibodies with the peptide antigen (1:10; Oncogene Science). As a positive control in immunohistochemical studies, we used paraffin slides of the invasive breast carcinoma.

In immunocytochemical studies, we used SKBR-3 cells as a positive control (*c-ebB-2*–positive breast carcinoma cell line.[6,21] As a negative control we used colon cancer cell line SW480 (colon cancer that had shown earlier that it does not express *c-erbB-2* protein[22]).

Statistical Analysis

Categorical data were analyzed using χ^2 statistics. The probability of survival was calculated for the different subgroups by the Kaplan–Meier method. Four-week mortality was excluded from the survival curves. Statistical differences were evaluated by the Mantel–Cox test. All evaluations were performed using BNDP Statistical Software (Cork, Ireland). Multivariate analysis was performed with Cox's proportional hazards model. A stepwise procedure was used to identify the major prognostic indicators independently associated with survival.[23–25]

Results

Specificity of Antibodies for p185$^{HER-2/neu}$

A series of specific antipeptide antibodies for the HER-2/*neu* gene product have been described and shown not to cross-react with the closely related proteins. However, to verify the specificity of the MAbs used in these experiments, we tested them immunocytochemically on a human breast carcinoma cell line SK-BR-3 and on human colon carcinoma cell lines HT-29 and SW480 (Figure 1). The cell lines SK-BR-3 (Figure 1*A*) and HT-29 (Figure 1*B*) have been previously shown to overexpress HER-2/*neu* protein.[6,21] As a negative control, we used a colon adenocarcinoma cell line SW480 (Figure 1*C*), in which this protein was not expressed in measurable quantities.[22] Confirmation of the specificity of antibodies was established by immunoprecipitation of cell lysates and Western blot analysis that in both cases showed precipitation of a single band at 185 kilodaltons. As shown in Figure 2, p185$^{HER-2/neu}$ was detected in cell lysates of SK-BR-3 and HT-29 cells but not in SW480 cells. To verify that the antibodies used did not cross-react with molecules structurally related to p185$^{HER-2/neu}$, we also performed Western blot analysis with antibodies specific for HER-2/*neu*, EGF receptor, and HER-3/*erbB-3* product that followed immunoprecipitation of cell lysates with HER-2/*neu*–specific antibodies. Our results showed that HER-2/*neu*–specific antibodies do not recognize structurally similar proteins, namely, EGF receptor and HER-3/*erbB-3* (data not shown).

Paraffin slides of invasive breast carcinoma were used as a positive control for immunohistochemistry and showed a strong, predominantly membrane-bound immunoreaction (Figure 1*D*). Control staining on paraffin sections of colon adenocarcinomas was performed by omitting the primary antibody, with no immunoreaction observed (Figure 1*E* and *F*).

p185$^{HER-2/neu}$ in Colorectal Lesions

Two hundred twenty-one colon samples (45 samples of normal colon and 176 samples of different colon neoplastic lesions) were examined for the presence of HER-2/*neu* oncoprotein (Table 1). Normal mucosa, polyps, adenomas, and adenocarcinomas of colorectal origin involved in our study clearly differed in the intensity and

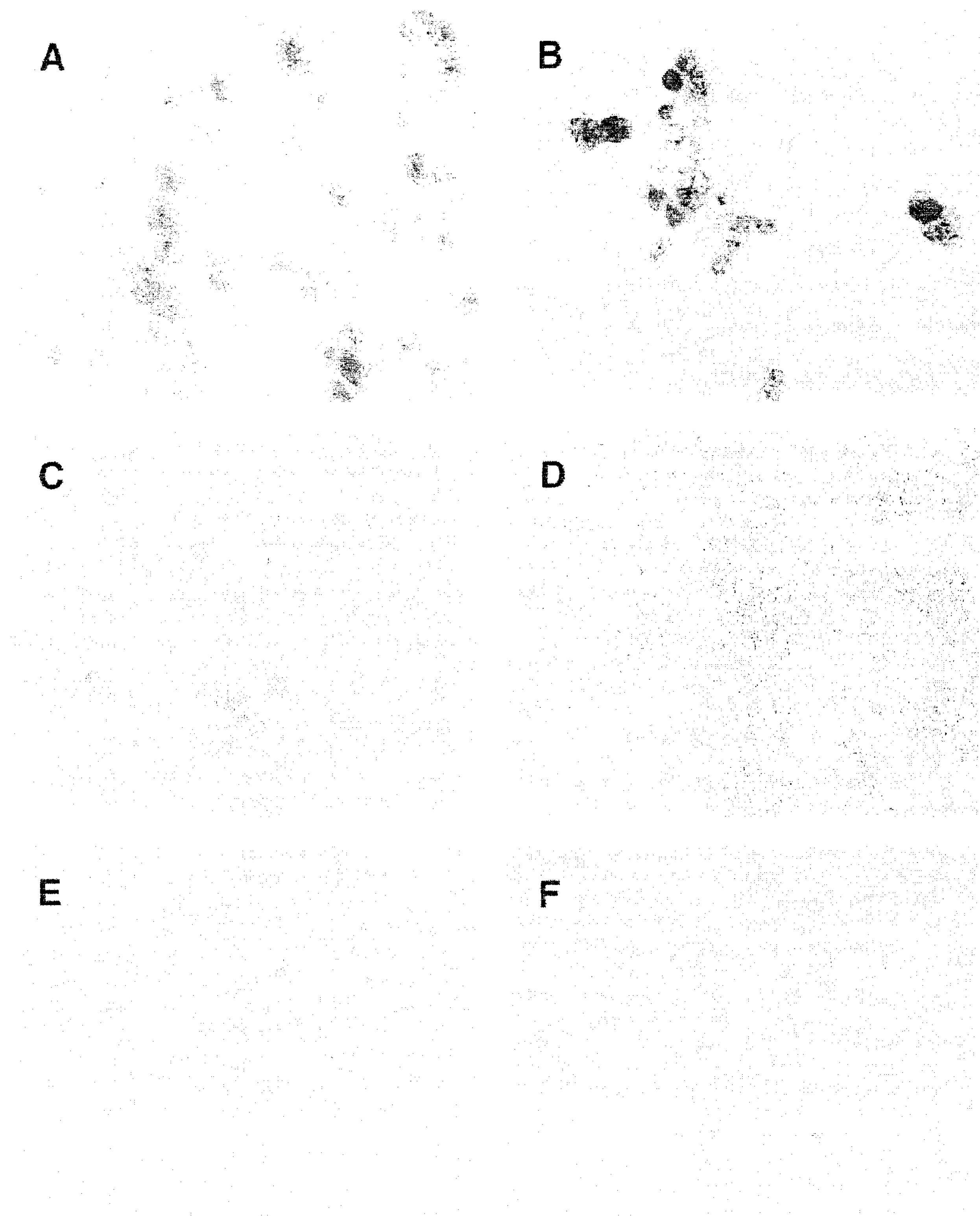

Figure 1. p185$^{HER-2/neu}$ immunostaining in (*A*) human breast carcinoma cell line SK-BR-3, (*B*) human colon carcinoma cell line HT 29, and (*C*) SW480 cells using MAb *c-neu* (Ab-3; Oncogene Science). SK-BR-3 cells showed predominantly membrane staining, HT 29 cells showed mixed membrane and cytoplasmic staining, and SW480 cells showed no staining. (*D*) p185$^{HER-2/neu}$ immunostaining in human breast carcinoma. Immunohistochemistry was performed on a formalin-fixed, paraffin-embedded tissue specimen. (*E*) p185$^{HER-2/neu}$ immunostaining in human colon adenocarcinoma. Tumor cells showed cytoplasmic staining. (*F*) Negative control of the same specimen. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue specimens (original magnifications: *A–C*, *E*, and *F*, 1000×; *D*, 400×).

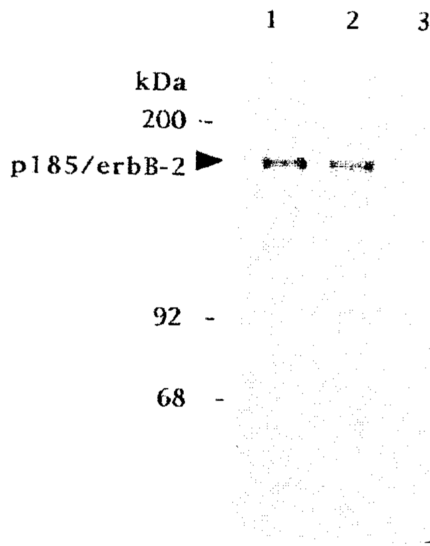

Figure 2. Immunoprecipitation of cell lysates followed by Western blot analysis with *c-neu* (Ab-3) antibodies showed a single band at 185 kilodaltons in cell lysates of SKBR-3 (*lane 1*) and HT-29 (*lane 2*) cells but not in SW480 (*lane 3*) cells.

histological localization of HER-2/*neu* immunostaining (Figure 3).

Normal colon. The most histologically normal colon samples examined (24 of 29) were negative for HER-2/*neu* oncoprotein. One of the samples showed a weak and four of them moderate reaction. Moderately stained samples were histologically normal colon mucosa adjacent (within 5 cm from the tumor) to adenocarcinoma. Interestingly, normal mucosa adjacent to adenomas did not express immunohistochemically detectable amounts of HER-2/*neu* product.

Hyperplastic polyps. Hyperplastic polyps showed the lowest rate of positivity for HER-2/*neu* protein with weak immunoreaction in 50% of cases (8 of 16) and moderate immunoreaction in 25% of cases (4 of 16). The staining pattern was essentially identical in all positive cases. The granular immunoreaction was associated with the cytoplasmic membrane together with intracytoplasmic staining in the basolateral region of luminal epithelial cells and no staining in maturing crypt cells and proliferating basal cells (Figure 3*A* and *B*).

Adenomas. Neoplastic progression in the colon is characterized by the appearance of tumors defined histologically as tubular, tubulovillous, and villous adenomas. This study included sections from 21 such lesions (Table 1). Tubular adenomas showed a moderate $p185^{HER\text{-}2/neu}$ immunoreaction in >50% of cases with the staining pattern similar to the one seen in hyperplastic polyps. Nevertheless, in adenomas, $p185^{HER\text{-}2/neu}$ localization was not restricted only to luminal cells of the colon but also involved the cells located deep in the crypt (Figure 3*C*). Tubulovillous and villous adenomas were moderately (2 of 4) or strongly (2 of 4) positive for HER-2/*neu* oncoprotein. However, a more significant finding was the pattern of staining. This pattern was clearly different from that in tubular adenomas and resembled one observed in most of the HER-2/*neu*–positive colorectal adenocarcinomas (see further in the text).

Adenocarcinomas. Adenocarcinomas of the colorectum were significantly more positive for the HER-2/*neu* protein than benign and premalignant lesions involved in this study. Of 152 adenocarcinomas, only 25 (16%) showed a weak immunostaining and the other samples were either moderately (41%) or strongly (43%) positive. The observed staining pattern was essentially identical in all carcinomas and was represented by the intense mixed membrane and cytoplasmic staining of tumor cells with negative surrounding normal tissue. Most of the tumor cells were stained evenly, and no polarity was noted in general (Figure 3*D* and *E*). In all cases, staining was computed out by the appropriate control experiments (Figure 1*F*). In four cases (described above) we noticed moderate HER-2/*neu* expression in normal tissue adjacent to carcinoma. In these cases, although the tissue was histologically normal, localization of the HER-2/*neu* protein extended into cells of the crypt in a way similar to adenomas. By using anti–$p185^{HER\text{-}2/neu}$ antibodies we were also able to show tumor cells located in the submucosa of $p185^{HER\text{-}2/neu}$–positive colorectal carcinomas. These cells were strongly positive compared with the negative surrounding normal tissue (Figure 3*F*).

Analysis of $p185^{HER\text{-}2/neu}$ in the regional lymph node metastasis (Figure 3*G*) has provided additional information of biological and clinical interest. The patients we

Table 1. Immunohistochemical Reactivity of Anti–*c-erbB-2* MAb in Normal Colon, Adenomas, and Adenocarcinomas

Histological type	No. positive/ no. tested	Staining (*% positive cases*) +	++	+++
Normal colon	5/29	1 (3)	4 (14)[a]	0
Hyperplastic polyp	13/16	8 (50)	4 (25)	1 (6)
Tubular adenoma	15/17	4 (24)	9 (53)	2 (12)
Tubulovillous adenoma	4/4	0	2 (50)	2 (50)
Adenocarcinoma	155/155	25 (16)	63 (41)	67 (43)

NOTE. χ^2 statistics showed that reactivity (staining) is highly dependent on histological type of lesion (total $\chi^2 = 26.984$; $P < 0.001$, *df* = 8).
[a]Normal tissue adjacent to carcinoma.

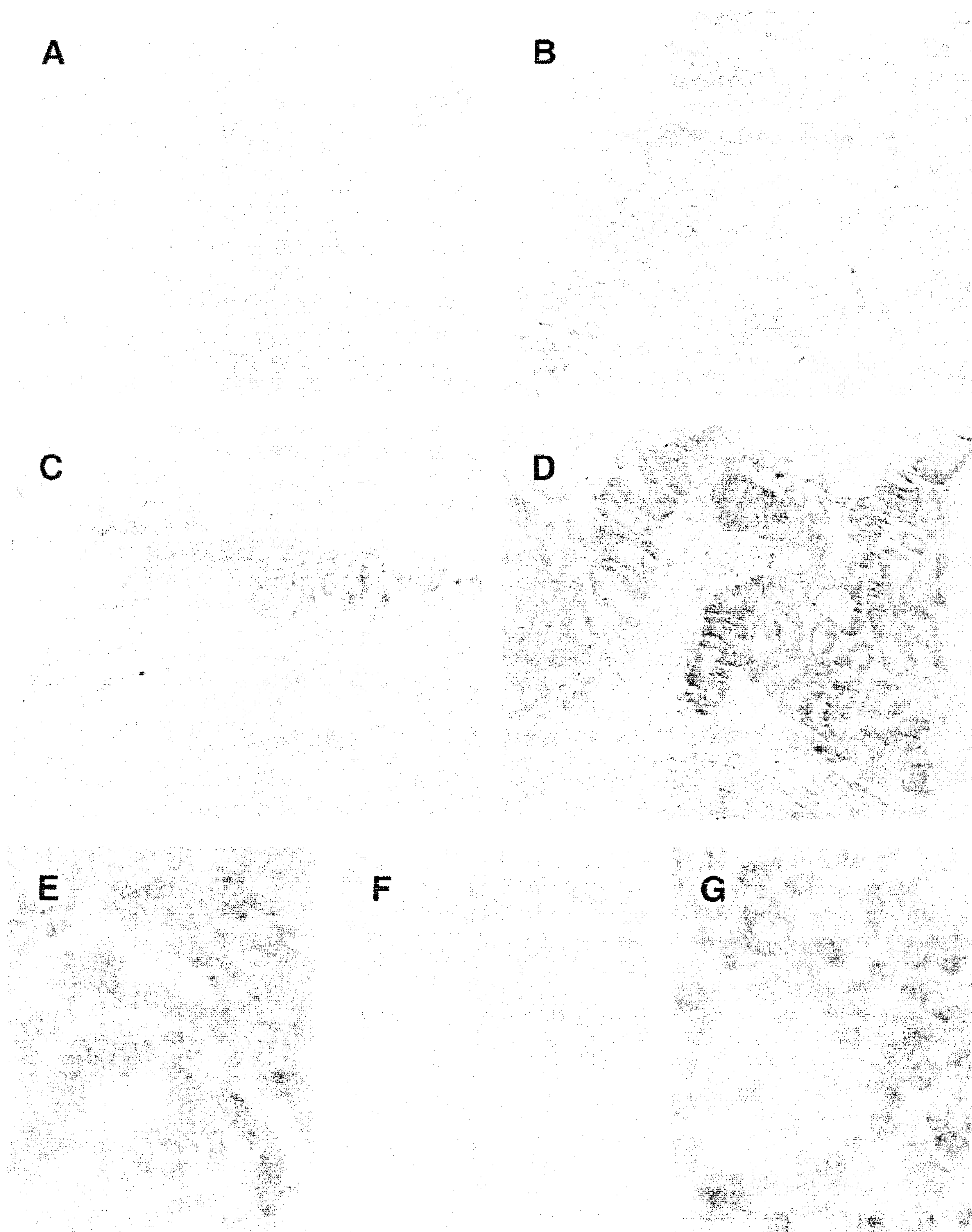

Figure 3. p185$^{HER-2/neu}$ immunostaining in human (*A–C*) benign colon lesions, (*D* and *E*) colon adenocarcinomas, and tumor cells in the (*F*) submucosa and (*G*) regional lymph node (under the immersion objective) using MAb *c-neu* (Ab-3). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue specimens (original magnifications: *A* and *D*, 250×; *B*, *C*, *E*, and *F*, 1000×).

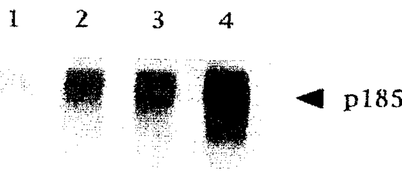

Figure 4. Immunoprecipitation of the colon carcinoma sample lysates with the anti–p185$^{HER-2/neu}$ antibodies: SKBR-3 cells (*lane 1*) and the tumor samples that immunohistochemically showed weak (*lane 2*), moderate (*lane 3*), and strong (*lane 4*) positivity.

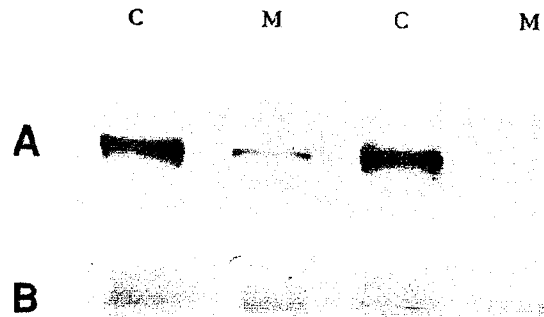

Figure 5. (*A*) Immunoprecipitation of the two colon carcinoma samples, cytosol (C) and membrane (M) proteins with p185$^{HER-2/neu}$ antibodies, (*B*) followed by Western blot analysis using P-Tyr (Ab-2) antibodies.

tested (whose primary tumors were strongly positive for HER-2/*neu* protein) also had HER-2/*neu*–positive tumor cells in the regional lymph nodes. Depending on the case, the size of the metastasis ranged from multicell structures (also distinguishable by the regular pathohistological examination) to a few positive cells (two cases, previously not recognized as metastasis positive, but with greatly shortened metastasis-free period). In all cases, the tumorigenic cells were strongly positive and easily recognizable among negative lymphocytes and stroma.

Immunoprecipitation

Immunoprecipitation of lysates of colon carcinoma samples with the specific anti–p185$^{HER-2/neu}$ antibodies resulted in the precipitation of a single band at 185 kilodaltons. The quantity of immunoprecipitated proteins varied among different samples and correlated with the level of immunoreaction obtained through the immunohistochemical analysis. In the example shown in Figure 4, immunoprecipitation of the tumor sample that was strongly positive for HER-2/*neu* protein showed precipitation of a much stronger band (lane 4) than the immunoprecipitation of lysates from moderately (lane 3) or weakly (lane 2) positive tumors.

To verify results obtained by immunohistochemistry we also performed separate analysis of cytosol and membrane preparations of colon cancer samples with p185$^{HER-2/neu}$ antibodies. These results showed that p185 was present in both the membrane and cytosol fraction. This protein in both fractions also showed phosphotyrosine activity (Figure 5).

HER-2/*neu* Expression and Clinical Parameters

Survival analysis. Exploratory analysis was conducted to correlate the outcome of patients monitored during the 260-week period with p185$^{HER-2/neu}$ immunoreactivity results. Survival analysis was performed on 119 patients who survived for >4 weeks after surgery. The survival curves according to HER-2/*neu* expression are shown in Figure 6. The median survival time of the patients with weak HER-2/*neu* oncoprotein expression was 209 weeks with >50% of them alive at the end of the monitoring period. The median survival time of the patients with moderate and strong HER-2/*neu* oncoprotein expression was 120 and 28 weeks, respectively. Statistical analysis showed that patients with tumors that were weakly stained for HER-2/*neu* protein had significantly longer survival ($P < 0.05$) than patients with moderately or strongly positive tumors. A significant difference was also observed between moderately and strongly positive tumors ($P < 0.01$).

Histological grading. No correlation was found between the rate of HER-2/*neu* gene expression and the grade of positive adenocarcinoma samples ($P > 0.05$).

Dukes' staging. The rate of HER-2/*neu* gene expression closely correlated with Dukes' staging of positive adenocarcinoma samples (Table 2). Tumors that were staged as Dukes' A and B showed weak or moderate p185$^{HER-2/neu}$ immunostaining, whereas tumors of pa-

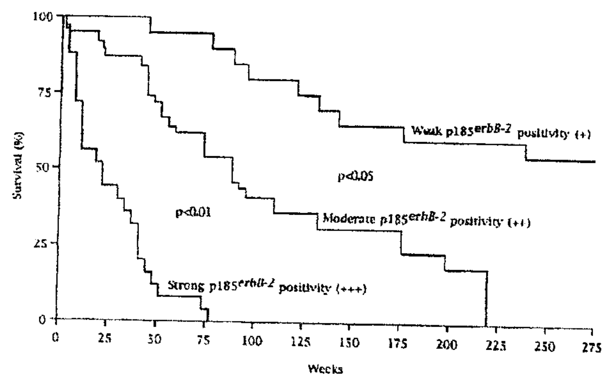

Figure 6. Survival of 119 patients with p185$^{HER-2/neu}$–positive colorectal cancer stratified by the intensity of p185$^{HER-2/neu}$ immunostaining.

Table 2. Relationship of c-*erbB*-2/HER-2 Staining to Dukes' Stage

Dukes' stage	No. of cases	c-*erbB*-2/HER2 staining		
		+	++	+++
A	34	13	18	3
B	40	10	20	10
C	77	10	26	41

NOTE. χ^2 statistics showed that staining is highly dependent on Dukes' stage of tumors (total χ^2, 26.984; $P < 0.001$; $df = 8$).

tients with confirmed metastases in regional lymph nodes (Dukes' C) or in distant organs (Dukes' D) in most cases showed moderate or strong immunoreaction at the time of surgery.

Metastasis-free disease. The correlation was found between HER-2/*neu* overexpression and the time of liver metastases detection. Patients with tumors that showed a weak expression of investigated oncoprotein had a significantly longer metastases-free period than those bearing tumors with moderate or strong expression ($P < 0.01$); the difference between patients with moderately and strongly positive tumors for p185[HER-2/neu] was also significant ($P < 0.01$; Figure 7).

Interestingly, patients with tumors staged as Dukes' A and/or B that were rated as moderately or strongly p185[HER-2/neu] positive at the time of surgery, in most cases ([illegible]) developed distant metastasis during the monitoring period and had a significantly shorter survival ($P < 0.05$) than patients with weakly positive tumors. The median survival time of these patients was [illegible] weeks with 38.6% still alive at the end of the monitoring period.

Regression analysis. Table 3 shows the survival of 80 patients stratified by a range of clinical and pathological features. A Cox proportional hazards model identified age, Dukes' stage, and intensity of *erbB-2* staining as having prognostic importance (Table 3). Stepwise regression analysis confirmed that Dukes' stage, age, and intensity of *erbB-2* staining were independently related to survival (Table 4).

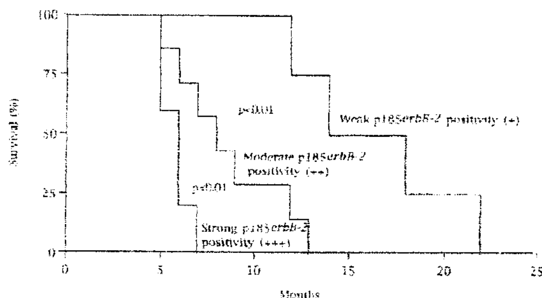

Figure 7. Metastasis-free period of patients with HER-2/*neu*–positive colorectal tumors correlated with the intensity of p185[HER-2/neu] immunostaining.

Table 3. Survival of 80 Patients With Colorectal Cancer Stratified by Clinicopathologic Features

	No. of cases	5-yr survival (%)	Regression analysis: Relative risk of death	Regression analysis: *P* value
Age (*yr*)				
<70	43	63	1	
≥70	37	32	2.20	0.008
Sex				
Male	43	42	1	
Female	37	57	0.73	0.32
Tumor size (*cm*)				
≤5	55	44	1	
>5	25	60	0.69	0.29
Histological grade (differentiation)				
Well	11	64	1	
Moderate	36	56	1.33	0.53
Poor	33	33	2.03	0.16
Dukes' stage				
A	21	81	1	
B	25	44	3.60	0.01
C	34	32	5.15	0.0005
erbB-2 staining				
+	22	73	1	
++	40	43	2.44	0.03
+++	18	33	4.68	0.008

Discussion

Amplification and overexpression of the HER-2/*neu* gene have been reported in adenocarcinomas of various origin, suggesting the importance of this oncogene in the neoplastic process. However, despite the large number of studies performed, details on the role of the HER-2/*neu* protein in the carcinogenesis remain vague. In some adenocarcinomas, particularly in breast and stomach, its overexpression closely correlates with the overall clinical course of the disease and survival of patients.[45–47] In this study, we analyzed the expression of HER-2/*neu* gene product in normal human colon tissue and in samples representing different stages of neoplastic progression. We show that normal colon tissue and benign and malignant colon lesions clearly differ in the

Table 4. Stepwise Regression Model

	β	SE	RR	*P* value
Dukes'	0.6123	0.219	1.845	0.0052
Age	0.8706	0.3223	2.388	0.0071
erbB-2 staining	0.6113	0.2496	1.843	0.0143

RR, relative risk.

rate of HER-2/*neu* protein expression and its intracellular localization and that, in colorectal cancer, the level of HER-2/*neu* expression correlates with the relapse-free period and postoperative survival time. Furthermore, using the relevant statistical methods, we show that together with the age of the patients and Dukes' stage, the expression of p185$^{HER-2/neu}$ is an independent prognostic factor in p185$^{HER-2/neu}$–positive colorectal cancers.

Although some reports suggest that the amplification of HER-2/*neu* gene in colorectal carcinoma is not a very frequent event,[6,22,26] we found that a significant number of adenomas and adenocarcinomas overexpress HER-2/*neu* protein. This difference is not surprising because it is known that HER-2/*neu* overexpression may occur via a number of different mechanisms[21,27,28] and is not only a result of gene amplification. The histological as well as intracellular distribution of HER-2/*neu* product that we observed was similar to that previously described in colorectal lesions.[22] In normal colon tissue and polyps, p185$^{HER-2/neu}$ was localized in the luminal surface cells with no crypt involvement. The immunostaining was associated with both cytoplasm and membrane, with the basolateral region of the apical cells staining most intensively. Tubular adenomas showed a similar pattern of immunostaining. However, in these cases, the staining was stronger, less polarity was noted, and the localization of HER-2/*neu* protein extended into the cells of the crypt. Interestingly, a similar picture was also observed in the normal tissue adjacent to carcinoma (but not adjacent to adenoma). D'Emilia et al.,[22] who also noted this, analyzed the expression of p185$^{HER-2/neu}$ in histologically normal tissue adjacent to carcinoma and showed that such aberrant distribution of the HER-2/*neu* product is restricted only to the regions located 5 cm proximal and distal from the tumor. This finding was confirmed by our results because the samples of the normal mucosa that showed positivity to p185 were those located within 5 cm from the tumor. All these results suggest the existence of alterations associated with compromised mucosal elements in this region, presumably as a result of contact with soluble molecules released from the tumor cells.

In the substantial number of the HER-2/*neu*–positive cases involved in this study, the cells showed membrane but also the intracytoplasmic immunostaining. This result is consistent with the number of other studies on various tumors and normal tissues.[19,20,22,29,30] However, these findings are a matter of numerous discussions because p185$^{HER-2/neu}$ has many properties of a "classical" growth factor transmembrane receptor[31,32]; therefore, it is expected to be localized predominately on the cell membrane. De Potter et al.[33] suggested that in breast lesions only a membrane staining should be considered as specific, whereas a cytoplasmic one is a result of cross-reactivity with a yet unknown 155-kilodalton protein located on the mitochondrial cristae of normal and malignant cells. However, our results showed that in the colon lesions this is not the case because immunoprecipitation and Western blotting of cell lysates with specific antibodies resulted in detection of a single band at 185 kilodaltons. Furthermore, separate analysis of cytosol and membrane preparations with p185$^{HER-2/neu}$ antibodies showed that only one specific band of the same size was present in both fractions. Peptides immunoprecipitated by anti–p185$^{HER-2/neu}$ antibodies from both fractions have been also recognized by anti-PTyr antibodies in Western analysis, implicating that in both cases anti–p185$^{HER-2/neu}$ antibodies immunoprecipitated a functionally active mature form of this tyrosine kinase receptor. These data are consistent with recent results by Xie and Hung[34] who showed that p185$^{HER-2/neu}$, on stimulation by its ligand, may be translocated to the nucleus where it exerts the transactivation function. Similarly to us, these investigators have shown phosphorylatically that p185$^{HER-2/neu}$ could be detected not only on cell membranes but also in the cytoplasm and in the nucleus of stimulated cells.

In agreement with evidence that intracytoplasmic staining is not an artifact is the finding that the intracellular distribution of immunoreactive p185$^{HER-2/neu}$ parallels the morphological and functional differences between normal cells of colonic mucosa and adenocarcinoma cells. In epithelial columnar-like cells of normal colonic mucosa and polyps p185$^{HER-2/neu}$ was localized in the basolateral regions of cells, but in adenomas, distinction in p185$^{HER-2/neu}$ staining pattern between basolateral and other regions of the cells was greatly reduced. Finally, in adenocarcinomas, this distinction is completely lost; the cells are stained evenly and no polarity could be noted. This finding is similar to that described by D'Emilia et al.[22] and agrees with the recent results of Kay et al.[19] who showed only intracytoplasmic *c-erbB-2* staining in colon carcinoma as well as those of Compiglio et al.[35] who showed colocalization (and possible physical interaction) of the p185$^{HER-2/neu}$ and integrin $\alpha_6\beta_4$ in lung carcinoma cells.

The behavior of colorectal cancer is believed to be dependent on multiple genetic events throughout the genome. Fearon and Vogelstein[36] have proposed the model of preferential sequence of mutational changes that is widely accepted as one of the dogmas of human tumor genetics. However, even these investigators have not excluded the possibility of existence of other alterations and suggested that accumulation of mutations is more important than the order in which they occur. The ques-

tion remains whether the altered level of HER-*2/neu* gene expression reflects a primary event in the generation of neoplastic lesions of the colon and, if not, on which stage in the multistep process of the colon tumorigenesis does it become important. Our results showed that overexpression and altered distribution of $p185^{HER-2/neu}$ could be detected in the early stages of the colon tumorigenesis.[17] This result is not unexpected because it has been already shown that HER-*2/neu* is, when overexpressed, capable of inducing malignant transformation in vitro[38,39] and in vivo.[illegible] Also, during human breast cancer development, activation of HER-*2/neu* seems to be one of the earliest events.[illegible]

On the other hand, this oncogenic product may also play its role in the advanced stages of certain cancers, especially by enhancement of metastatic potential through the induction of metastasis-associated properties of tumor cells. This feature has been suggested by the experimental evidence on 3T3 cells,[11] colorectal[15] and non–small cell lung cancer cell lines,[46] as well as transgenic animals.[illegible] These data have also been supported by our finding that patients bearing colorectal tumors with highly overexpressed $p185^{HER-2/neu}$ had a significantly shorter metastasis-free period than patients with low and moderately $p185^{HER-2/neu}$–positive tumors. Radinsky et al. showed that expression of EGF receptor by human colon carcinoma cells in vitro directly correlates with their ability to produce hepatic metastasis.[illegible]

Finally, one cannot exclude the possibility that HER-[illegible] sis. In such a scenario, the rate of $p185^{HER-2/neu}$ expression would be the result of increasing need of tumor cells (that have been transformed by the sequence of other genetic changes[36]) for additional growth-regulatory stimuli.

Regardless of whether the accumulation of the $p185^{HER-2/neu}$ is the cause or consequence of the carcinogenesis, our results show that the amount of this oncoprotein in $p185^{HER-2/neu}$–positive tumors correlates positively with the stage of the disease and postoperative survival time. Regression analysis confirmed that intensity of *erbB-2* staining was independently related to survival and could serve as a practical tumor marker. These results are in accordance with the recent study of Kay et al.,[19] who showed that cytoplasmic HER-*2/neu* protein [illegible] colorectal carcinoma. Further studies on the role of this oncoprotein in colorectal cancer will show whether it can [illegible]

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Received October 24, 1995. Accepted October 29, 1996

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