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Immunohistochemical Detection of p53 Protein, *c-erb*B-2 Protein, Epidermal Growth Factor Receptor Protein and Proliferating Cell Nuclear Antigen in Gastric Carcinoma

Woo Ick Jang, M.D., Woo Ick Yang*, M.D., Chong In Lee, M.D., Hyun Soo Kim, M.D., Kwang Seon Song, M.D., Mee Yon Cho*, M.D., Jong Koo Park,** M.D., Young Hak Shim, M.D.

Department of Internal Medicine, Pathology and Preventive Medicine***
Yonsei University Wonju College of Medicine

There is increasing evidence that genes involved in normal cell grwoth and differentiation (oncogenes) or genes that encode for growth factors are important in determining the development and biologic aggressiveness of gastric carcinoma. This study was undertaken to define the prognostic value of the overexpression of p53 protein, c-erbB-2 protein, EGFr protein and PCNA in gastric carcinomas. Using monoclonal antibodies, immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue sections from 84 primary gastric carcinomas. Overall, 34% of gastric carcinomas had nuclear-staining for p53 protein, 34% of carcinomas membrane staining for the c-erbB-2 and 74% of carcinomas membrane and cytoplasmic staining for EGFr, showing distribution in a heterogeneous fashion. PCNA was expressed as Grade 2 and 3 in 75% of patients with gastric carcinomas. Both c-erbB-2 and p53 staining was significantly associated with high grade expression of PCNA. p53 staining tended to be associated with positive nodal status and metastasis, and c-erbB-2 staining with positive nodal status only. Multivariate analysis using the Cox model showed that overexpression of p53 protein, c-erbB-2 protein and PCNA was not an independent prognostic variable in gastric carcinoma. These results suggest that expressions of p53 and c-erbB-2 protein are heterogeneous and that p53 and c-erbB-2 overexpressions are significantly associated with high proliferative activity in gastric carcinoma.

Key Words: *p53, c-erbB-2, Epidermal growth factor receptor, Proliferating cell nuclear antigen, Gastric carcinoma*

INTRODUCTION

There is increasing evidence that genes involved in normal cell growth and differentiation or genes that encode for growth factors play important roles in tumor development and in the biologic aggressiveness of various human cancers. The human gene for p53 is located on chromosome 17p and encodes a 393 amino acid nuclear phosphoprotein. The wind-type protein acts as a negative regulator of cell growth while paradoxically mutant proteins seem to activate a latent growth promoting or dominant oncogenic activity (Lane et al., 1992; Raycroft et al., 1990). Somatic mutation of p53 gene is found in breast cancer (Chen et al., 1991; Davidoff et al., 1991), lung cancer (Takahashi et al., 1989), colon cancer (Baker et al., 1989) and gastric cancer (Kim et al., 1991).

The c-erbB-2 oncogene encodes transmembrane glycoprotein with tyrosine kinase activity that is a putative growth factor receptor similar to, but distinct from

Address for correspondence: *Young Hak Shim, Department of Internal Medicine, Yonsei University Wonju College of Medicine, Ilsan-Dong, Wonju 220-740, Korea (Tel: 0371-41-6313)*

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the epidermal growth factor receptor (EGFr) (Coussens et al., 1985; King et al., 1985). Amplification of the c-erbB-2 gene is correlated with overexpression of its oncoprotein and is found in breast carcinoma (Alled et al., 1992; Corbett et al., 1990; Gasparini et al., 1992; Iglehart et al., 1990; Kim et al., 1991; Ro et al., 1989; Wright et al., 1989) and gatric carcinoma (Park et al., 1989; Falck et al., 1989; Yonemura et al., 1991).

Epidermal growth factor (EGF) plays an important role in cellular proliferation and differentiation. After binding to EGFr, EGF activates the tyrosine kinase subunit and autophosphorylation of the receptors. Overexpression of the EGFr protein is considered to be an important step in the autocrine stimulation of cancer cell growth and is found in lung cancer (Tateishi et al., 1990), breast cancer (Lewis et al., 1990; Tsutsumi et al., 1990), ovarian cancer (Scambia et al., 1992) and gastric cancer (Lee et al., 1991; Sugiyama et al., 1989; Yasui et al., 1988).

Proliferating cell nuclear antigen (PCNA) is a 36kd nuclear protein and is regarded as an auxillary protein of DNA polymerase-delta (Garcia et al., 1989; Kang et al., 1991: Kim et al., 1992). The expression of PCNA is correlated with the S-phase of the cell cycle. PCNA plays a critical role in the initiation of cell proliferation.

p53 mutant protein, *c-erb*B-2 protein and EGFr overexpression and PCNA expression may be used as prognostic markers in various cancers. Human gastric cancer seems to have a heterogeneous nature, so conventional pathologic factors are unable to predict accurately the prognosis for all patients. Therefore, using immunohistochemical studies of p53, *c-erb*B-2, EGFr and PCNA, we investigated the staining pattern, the relationship among p53 mutant protein expression, the *c-erb*B-2 protein overexpression, EGFr protein expression, expression of PCNA and conventional pathologic features to understand the biologic behavior and to predict their prognostic significance in gastric carcinoma.

MATERIALS AND METHODS

Patients

A retrospective study of 84 patients who underwent gastric surgery (70 cases) or gastroscopic biopsy (14 cases) at the Wonju Christian Hospital from 1981 to 1988 was included in the present study. Eighty-three cases were adenocarcinoma and only one case was adenosquamous carcinoma. Tumors were staged according to the criteria of the American Joint Committee of Cancer (3rd edition, 1988).

Immunohistochemical Analyses

Expressions of p53, *c-erb*B-2, EGFr protein and PCNA were demonstrated in 4-um sections of the routine formalin-fixed, paraffin-embedded blocks using monoclonal antibodies (NCL-p53-DO-7 for p53 protein, Novocastra Laboratories Ltd., UK: NCL-CB11 for *c-erb*B-2 protein, Novocastra Laboratories Ltd., UK: Anti-EGFR for EGFr protein, BioGenex Laboratories Ltd., USA: PC10 for PCNA, Dako Laboratories Ltd., Denmar). Sections were deparaffinized in 100% xylene and dehydrated through graded alcohols. The sections were preincubated with 30% hydrogen peroxide for 10 minutes to eliminate the background activity due to endogenous peroxidase activity, then washed with PBS (Phosphate Buffered Saline, pH7.6). Then the sections were preincubated with blocking sera to reduce nonspecific antibody bindings. Sections were incubated with NCL-p53-DO-7 for p53 protein at a dilution of 1:200, with NCL-CB11 for *c-erb*B-2 protein at a dilution of 1:50, with Anti-EGFR for EGFr protein at a dilution of 1:50 and with PC10 for PCNA at a dilution of 1:20. Slides were then incubated with biotinylated antimouse IgG and rinsed with PBS followed by streptavidin-biotin-peroxidase complex at a dilution of 1:100. The peroxidase reaction was developed using diaminobenzidine as chromogen. Sections were counterstained with Mayer's hematoxylin. Tumors were scord by assessing the site of staining (nuclear, membrane and/or cytoplasm) and the proportion of staining cells to tumor cells (Grade 0, 0%; Grade 1, 1 to 33%; Grade 2, 34% to 66%; Grade 3, 67% to 100%). Intensity of staining was not considered because the Universal immunostaining kit was highly sensitive. p53 staining was regarded as positive if any of the cells showed nuclear staining. *c-erb*B-2 staining was regarded as positive if Grade 2 and 3 staining was demonstrated. EGFr staining was regarded as positive if Grade 1, 2 and 3 was demonstrated. PCNA was divided into the low expressed group (Grade 1) and the high expressed group (Grade 2 and 3). Negative control sections were stained with omission of the primary antibodies. Scoring of p53 staining, *c-erb*B-2 staining, EGFr staining, PCNA staining and histologic variable assessment were made without prior knowledge of survival data. To determine whether each protein has a role on the biologic activity, we comparded each protein immunoreactivity with pathologic variables associated with prognosis: tumor size, serosal invasion, nodal status, number of positive nodes, metastasis, vascular and lymphatic permeation and stage.

Statistical Methods

Survival curves were prepared by the life table method, with comparisons between curves by the log rank test using a program provided by Professor SN Kim (College of Medicine, Konkuk University). Relationships between variables were examined by the Chi-square test. Multivariate analysis was performed using Cox's proportional hazards regression model of the SAS statistical package.

RESULTS

Immunohistochemical Stain of p53 Protein

Overall, 34% (29/84) of gastric carcinomas had nuclear staining for p53 protein. Among the twenty-nine positive cases ten were grade 1 positive, eight grade 2 and eleven grade 3. p53-positive cells were distributed in a heterogeneous fashion (Fig. 1). p53 staining tended to be associated with positive nodal status, metastasis and stage (Table 1).

Immunohistochemical Stain of *c-erb*B-2 Protein

Overall, 34% (29/84) of gastric carcinoma had membrane staining for *c-erb*B-2 protein (Fig. 2). Cytoplasmic staining of carcinoma was also occasionally associated. Normal cells of gastric mucosa rarely stained for *c-erb*B-2 protein. Among the twenty-nine positive cases nineteen were grade 2 positive, ten grade 3. Nineteen were grade 1 positive (this group was considered *c-erb*B-2 negative). *c-erb*B-2 positive cells were also distributed in a heterogenous fashion. *c-erb*B-2 staining tended to be associated with tumor size and positive nodal status (Table 1).

Immunohistochemical Stain of EGFr Protein

Overall, 74% (62/84) of gastric carcinoma had membrane and cytoplasmic staining for EGFr protein (Fig. 3). Among the sixty-two positive cases six were grade 1 positive, eleven grade 2 and forty-five grade 3. EGFr positive cells were also distributed in a heterogeneous fashion.

Table 1. Relationship between overexpression of p53/*c-erb*B-2 protein and conventional pathological variables in gastric carcinoma

	n	p53 (+) staining		*c-erb*B-2 (+) staining	
Total	84	29 (35%)		29 (35%)	
Tumor size					
>6cm	51	15 (29%)		14 (27%)	
<6cm	19	6 (32%)	NS	9 (47%)	NS[a]
Serosal invasion					
Negative	18	5 (28%)		5 (28%)	
Positive	61	21 (34%)	NS	21 (34%)	NS
Nodal status					
Negative	18	4 (22%)		3 (17%)	
Positive	53	17 (32%)	NS	20 (38%)	NS
No. of node					
<0, >10	32	12 (38%)		12 (38%)	
<10	21	5 (24%)	NS	8 (38%)	NS
Metastasis					
Negative	58	18 (31%)		18 (31%)	
Positive	26	11 (42%)	NS	11 (42%)	NS
V/L permeation[b]					
Negative	57	19 (33%)		21 (37%)	
Positive	26	10 (37%)	NS	8 (30%)	NS
Stage					
$I_a + I_b$	11	2 (18%)		1 (9%)	
II	12	3 (25%)		5 (42%)	
$III_a + III_b$	35	13 (37%)		12 (34%)	
IV	26	11 (42%)	NS	11 (42%)	NS

NS[a]: Statistically not significant

V/L permeation[b]: Vascular and lymphatic permeation

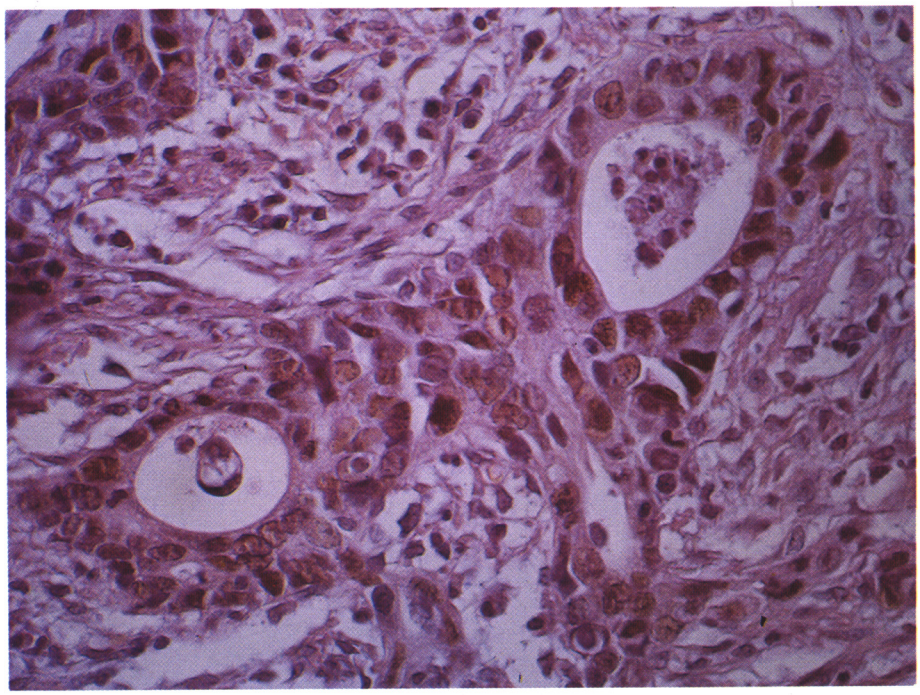

Fig. 1. Immunohistochemical staining of p53 protein in gastric carcinoma. The immunoreactivity for p53 protein is localized to the nucleus of the tumor cells (×400).

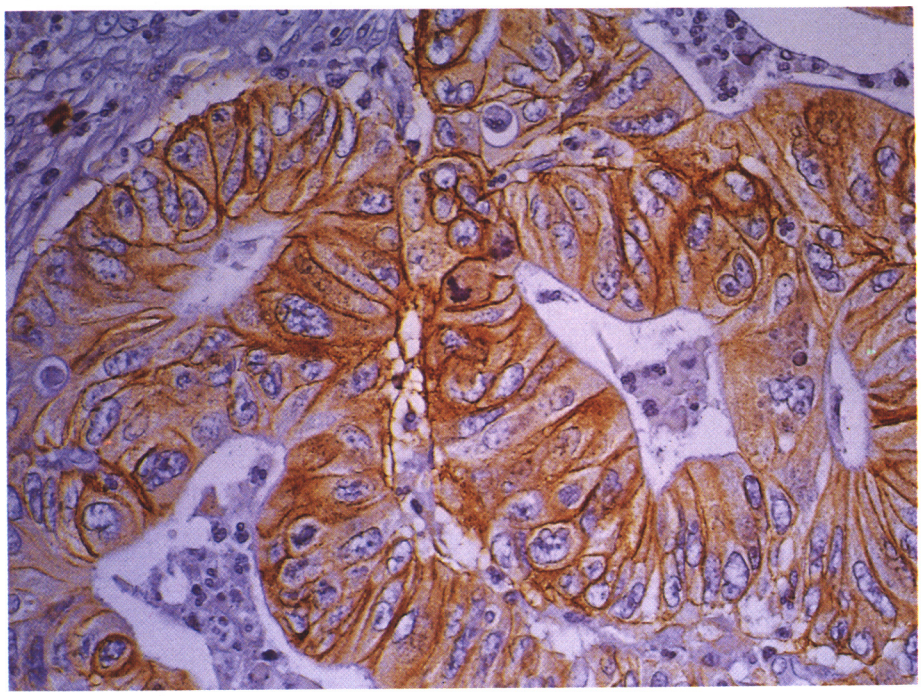

Fig. 2. Immunohistochemical staining of *c-erb*B-2 protein in gastric carcinoma. The immunoreactivity for *c-erb*B-2 protein is localized to the cell membranes of the tumor cells (×400).

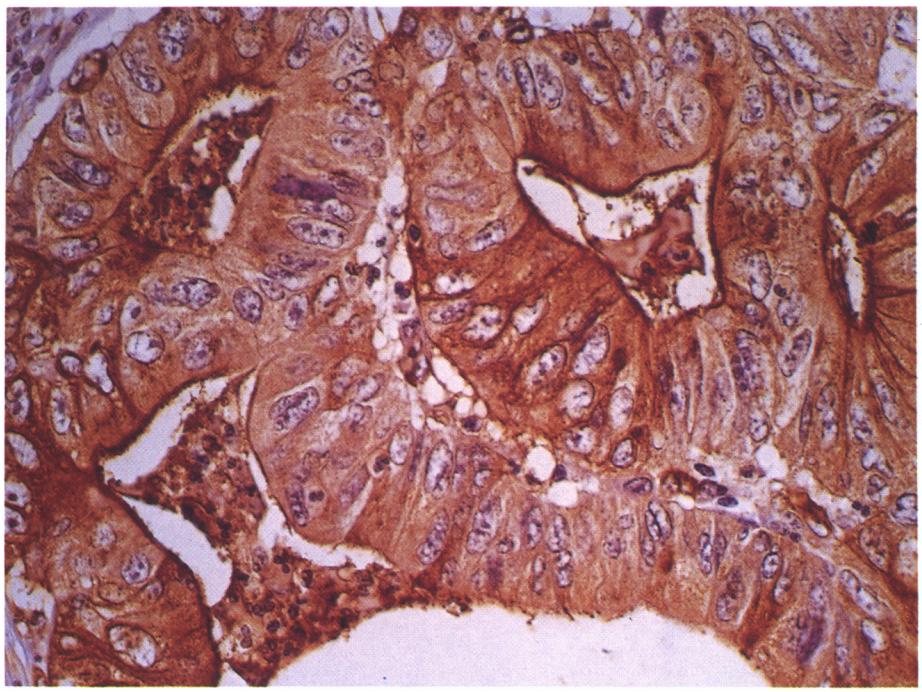

Fig. 3. Immunohistochemical staining of Epidermal growth factor receptor protein in gastric carcinoma. The EGFr was stained on cell membranes and on cytoplasms of the cancer cells (×400).

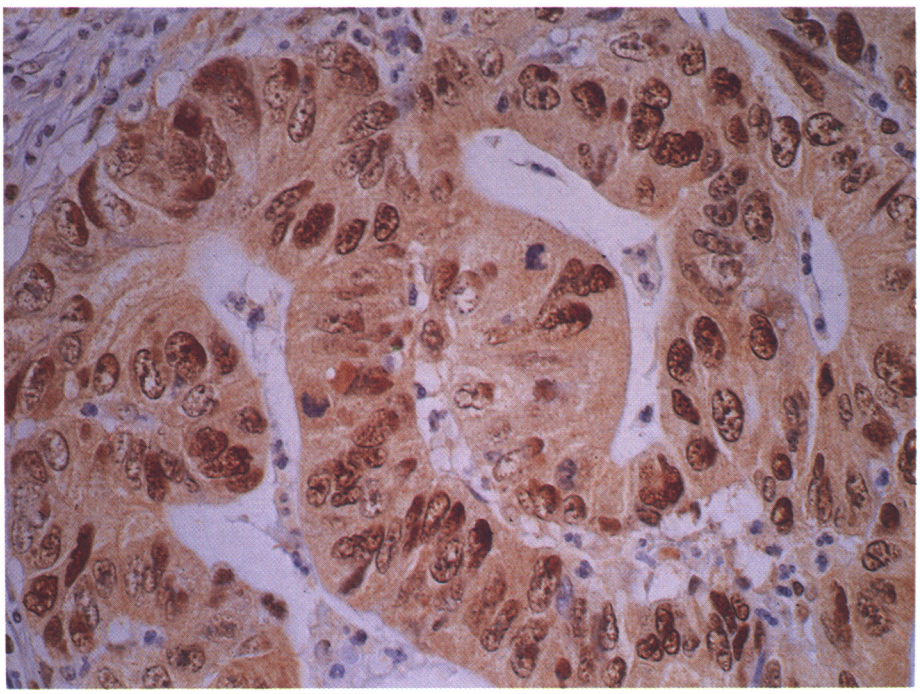

Fig. 4. Immunohistochemical staining of Proliferating cell nuclear antigen. Immunoreactivity for PCNA is localized to the nucleus of the tumor cells (×400).

Immunohistochemical Stain of PCNA

Nuclei of the gastric carcinoma cells were immunoreactive for PCNA in all cases (Fig. 4). Twenty-one were grade 1 positive, twenty-one grade 2 and forty-two grade 3.

Association of the Expression of p53 or *c-erb*B-2 protein with PCNA

Both p53 staining or *c-erb*B-2 staining were associated with high grade (Grade 2 and 3) expression of PCNA (Table 2).

Association of the Expression of *c-erb*B-2 protein with p53 protein and EGFr

Simultaneously positive and negative staining of *c-erb*B-2 and p53 was demonstrated in 17% (14/84) and in 48% (40/84) of gastric carcinomas respectively, so there was good agreement between the two factors (k coefficient=0.21, p=0.054) (Table 3). Simultaneous positive and negative staining of *c-erb*B-2 and EFGr was demonstrated in 27% (23/84) and in 19% (16/84) of gastric carcinomas respectively, so there was poor agreement between the two factors (k coefficient=0.06, p=0.40) (Table 3).

Univariate and Multivariate Analysis of Prognostic Variables in Gastric Carcinoma

Positive serosal invasion, positive nodal status, higher number of nodes (>10) and metastasis were individually associated with a significantly poorer overall survival. In our series p53 staining, *c-erb*B-2 staining and EGFr staining did not significantly affect the outcome of patients, but PCNA tended to affect it (Table 4). Kaplan-Meier survival curves by p53 status (Fig. 5). *c-erb*B-2 status (Fig. 6) and PCNA STATUS (Fig. 7) showed no significant survival differences between positive groups and negative groups or between grade 1 and grade 2-3. Considering a model contain-

Table 2. Relationship between overexpression of p53/*c-erb*B-2 protein and EGFr protein/PCNA in gastric carcinoma

	n	p53 (+) staining		*c-erb*B-2 (+) staining	
PCNA[a] staining					
Grade 1	21	4 (19%)		3 (14%)	
Grade 2 and 3	63	25 (40%)	p>0.05	26 (41%)	p>0.05
EGFr[r]					
Negative	22	6 (27%)		6 (27%)	
Positive	62	23 (37%)	NS[c]	23 (37%)	NS
*c-erb*B-2 staining					
Negative	55	15 (27%)			
Positive	29	14 (48%)	NS		
p53 staining					
Negative	55			15 (27%)	
Positive	29			14 (48%)	NS

PCNA[a]: Proliferating cell nuclear antigen
EGFr[r]: Epidermal growth factor receptor
NS[c]: Statistically not significant

Table 3. Association of overexpression of *c-erb*B-2 protein with expression of p53 and EGFr protein in gastric carcinoma

	n	k coefficient	p-value
*c-erb*B-2 and p53 staining		0.21	0.054
Both negative	40 (48%)		
Either positive	30 (36%)		
Both positive	14 (17%)		
*c-erb*B-2 and EGFr staining		0.06	0.40
Both negative	16 (19%)		
Either positive	45 (54%)		
Both positive	23 (27%)		

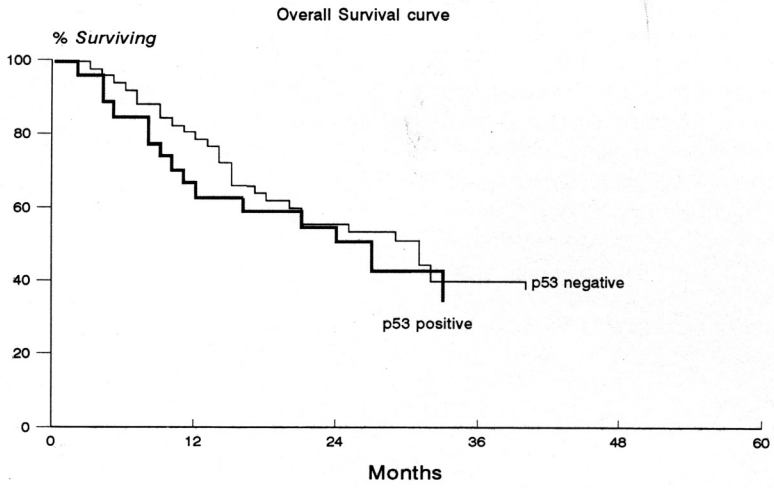

Fig. 5. Five-year overall survival in p53 negative (−) and p53 positive (−) patients.

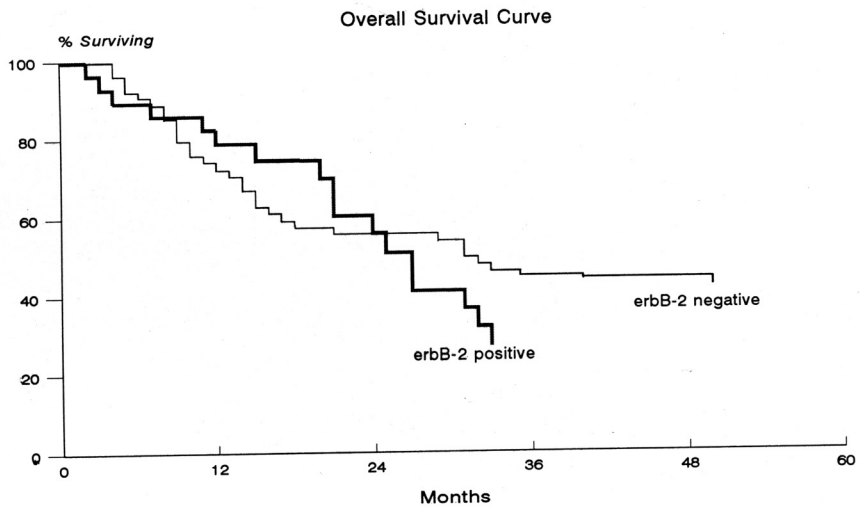

Fig. 6. Five-year overall survival in c-erbB-2 negative (−) and c-erbB-2 positive (−) patients.

ing serosal invasion, positive nodal status and metastasis, serosal invasion and positive nodal status retained a significant predicitve value (Table 4).

DISCUSSION

Serosal invasion, positive nodal status and metastasis are regarded as some of the most important prognostic variables in gastric carcinoma. The number of nodes is another important prognostic factor as well

as the group of node even though the higher group (N_3) node involvement cases tend to have the greater numbers of nodes. Because the outcome of patients is quite different in spite of the same stage, there have been a lot of trials to seek new biological indicators of tumor aggressiveness potentially useful in predicting the outcome of patients with gastric carcinoma. DNA ploidy, proliferative activity, expression of growth factor or receptor (Epidermal growth factor or receptor), amplification of various protooncogenes (including c-myc, c-Ha-ras, c-fps and *c-erb*B-2) (Lee et al., 1989; Yonemura et al., 1991; Yoon et al., 1989) and recently tumor suppressor gene (p53) are now regarded as potential biological indicators of gastric carcinoma. Especially, great interest has been concentrated on the biological role of overexpression of *c-erb*B-2 oncoprotein and accumulation of p53 oncoprotein.

p53 is a putative tumor suppressor gene,negatively regulating the cell cycle and requiring loss-of-function mutations for tumor formation: wild-type p53 protein suppresses the transformation of cells in culture by other oncogenes, the growth of transformed cells in culture, and the tumorigenic potential of cells in animals (Levine et al., 1991). Loss of normal p53 tumor suppressor function was resulted from genomic rearrangement, homozygous deletion and loss of heterozygosity (LOH) with concomitant point mutaion of the remaining allele. Recent investigations have suggested that

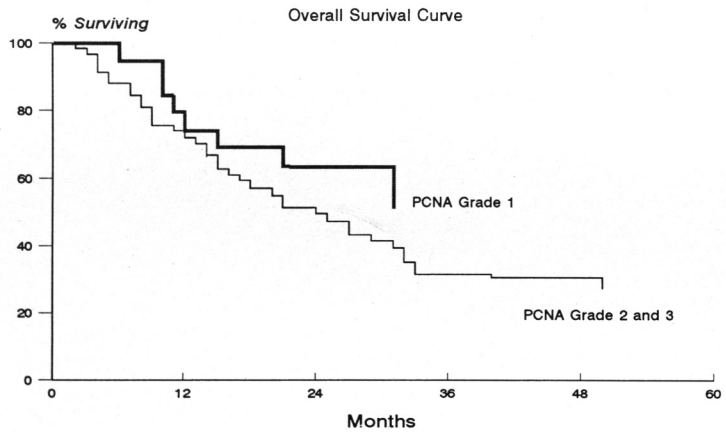

Fig. 7. Five-year overall survival in PCNA Grade 1 (−) and PCNA Grade 2 and 3 (−) patients.

Table 4. Prognostic factors of gastric carcinoma

	Univariate Scores α^2	analysis p	Multivariate Wald α^2	analysis p
Tumor size	1.98	0.16		
Serosal invasion	5.20	0.02	4.35	0.03
Nodal status	7.21	0.007	6.09	0.01
Number of nodes	12.20	0.0005		
Metastasis	5.01	0.02		
p53 expression	1.01	0.32		
*c-erb*B-2 overexpression	0.42	0.52		
EGFr[a] expression	1.74	0.19		
PCNA[b] high grade	3.22	0.77		

EGFr[3]: Epidermal growth factor receptor
PCNA[b]: Proliferating cell nuclear antigen
Note: Number of nodes was not considered for multivariate analysis.

mutated p53 gene product, in spite of absence of LOH, may contribute to the malignant phenotype by inactivating normal p53 function in a dominant-negative fashion (Vogelstein, 1990).

Immunohistochemical study using human specific anti-p53 monoclonal antibody (PAb1801) is positive only in cell lines or in tissue with p53 mutations resulting in alterations of protein conformation and prolonged half-life of mutant p53 proteins. In general, mutant forms of p53 protein that are transforming have several properties that distinguish them from wild type. They do not bind to the simian virus 40 tumor antigen but to form stable complexes with hsc70, a constituitively expressed member of the heat shock family (Finaly et al., 1988). Mutant p53 protein-hsc70 complex results in an increased half-life of mutant p53 protein. Normal p53 protein is virtually undetectable histologically because of its short half life.

Morkeve and Laerum (1991) and Thor et al. (1992) demonstrate that immunohistochemical stains with formalin-fixed, paraffin-embedded tumors can give results comparable with those with fixed, frozen tissue sections even though there is a report showing a higher percentage of p53-staining using frozen tissue (Bartek et al., 1990).

Variability of immunohistochemical staining for p53 protein may be related with cell cycle variation in p53 protein (Levine et al., 1991; Bischoff et al., 1990), Morkve and Laerum (1991) demonstrated that some tumors showed increased p53 during G_1, S and G_2M phases and aneuploidy populations showed a uniformly high p53 content.

The type of p53 gene mutation is correlated with the expression of mutant p53 protein. Bodner et al. (1992) reported that lung cancer cell lines with definite overexpression of p53 protein by immunohistochemistry had p53 missense mutations in exon 5-8, while cell lines with low or no expression of p53 protein had deletions, splicing mutation, nonsense mutation and missense mutations outside of exon 5-8.

p53 gene abnormalities including missense mutation, deletion resulting in a frame shift and intronic point mutation had been reported in gastric carcinoma (Kim et al., 1991). So gastric cancer cells with deletion, intronic point mutation and missense mutation outside of exon 5-8 possibly may be unstained by immunohistochemistry using anti-p53 monoclonal antibodies.

Our data showed 34% of gastric carcinomas had nuclear staining for p53 protein and these results are comparable with colon carcinoma (47%-55%) (van den Berg et al., 1989), breast carcinoma (15.5-45.5%) (Bartek et al., 1990; Cattoreti et al., 1988; Sommer et al., 1992) and lung cancer (55%) (Iggo et al., 1990). p53 staining tended to be associated with positive nodal status and metastasis without statistical significance.

Even though advanced staged gastric cancer have higher positive rates of p53 staining, 18% of stage I-gastric cancer was also stained. This means that p53 mutations can occurr at the early of gastric cancer. Thirteen to twenty-two percent frequencies of p53 accumulation have been noted in in-situ carcinomas of the breast (Bartek et al., 1990; Davidoff et al., 1991; Thor et al., 1992).

Whether immunohistochemically detected p53 accumulation can be used as an independent prognostic variable is still pending, p53 protein staining was reported to be an independent prognostic variable in axillary node negative and positive breast cancers (Thor et al., 1992). But Ostrowski et al. (1991) reported that p53 protein expression may be a marker of more aggresive carcinomas but that the prognostic power of expression is likely to be weak and unlikely, therefore, to be of clinical value.Isola et al. (1992) reported tat overexpression of the p53 and *c-erb*B-2 protein indicates a high malignant potential in axillary node negative breast cancer, but it is not a significant prognostic factor indenpendent of the cell proliferation rate. Our data showed that p53 staining tended to be associated with cell proliferation rate, positive lymph node status and metastasis, but overexpression of p53 protein was not an independent prognostic factor by multivariate analysis using the Cox model.

Although there is still some controversy, amplification or overexpression of *c-erb*B-2 is regarded as a new biological indicator of tumor aggressiveness potentially useful in predicting the outcome of patients with breast carcinoma (Allred et al., 1992; Gasparini et al., 1992; Wright et al., 1989). Overexpression *c-erb*B-2 protein is also an important independent prognostic marker for gastric cancer (Yonemura et al., 1991). Yonemura et al. (1991) demonstrated that high malignant potential of tumors with *c-erb*B-2 protein expression may be closely associated with the potential for lymph node metastasis. But our results showed that *c-erb*B-2 staining tended to be assoicated with cell proliferation rate and positive lymph node status, but overexpression of *c-erb*B-2 was not an independent prognostic factor by multivariate analysis using the Cox model.

Since *c-erb*B-2 protein and EGFr protein are glycoprotein receptors with similar structures, we investigated their agreement. Our results showed that there was a very poor agreement between the two factors (k coefficient = 0.06, p = 0.40). Our findings showed

mutually independent expression of the two closely related protooncogenes. These results are the same as were reported in breast cancer (Gasparini et al., 1992; Tsutumi et al., 1990; Yonemura et al., 1991). Even though the *c-erb*B-2 protein and the p53 protein are structurally different, there was a relatively good agreement between the two factors (k coefficient=0.21, p=0.054). These two factors were also significantly associated with cell proliferation rate. These results are the same as those reported in breast cancer (Isola et al., 1992).

EGFr protein was stained in the cell membrances and partly in the cytoplasm. It was suspected that EGFr protein stained in the cytoplasm may be internalized complexes of EGF and EGFr (Sugiyama et al., 1989) Immunohistochemically, EGFr immunoreactivity was detected in 32% to 50% in gastric carcinoma (Sakai et al., 1986; Sugiyama et al., 1989; Yasui et al., 1988). Our data showed a somewhat higher incidence of EGFr staining (74%), even though no clear reason for this was ellucidated.

Isola's finding (1992) of an increased S-phase fraction in primary breast carcinomas with p53 protein overexpression strongly suggests that overexpressed p53 proteins interfere with normal cell cycle regulations and confer a proliferative advantage to the cancer cells in vivo. Caroretti et al. (1988) found a significant association between p53 immunoreactivity and the Ki-67 proliferation antigen. Borg et al. (1991) and Kallioniemi et al. (1991) have previously reported that *c-erb*B-2 protein overexpression is associated with an increased cell proliferation rate in breast cancer. Our data also demonstrated that p53 and *c-erb*B-2 staining were significantly associated with high grade expression of PCNA.

In summary, multivariate analysis using the Cox model showed that overexpression of p53, *c-erb*B-2 and PCNA was not an independent prognostic variable in gastric carcinoma. Expressions of p53 and *c-erb*B-2 protein are heterogeneous, and p53 and *c-erb*B-2 overexpressions are significantly associated with high proliferative activity in gastric carcinoma.

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