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Original Paper

Expression of p53 Protein as a Prognostic Factor in Patients With Gastric Cancer

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The prognostic value of overexpression of the p53-encoded protein was evaluated in 242 patients with gastric cancer. Formalin-fixed paraffin-embedded specimens of gastric adenocarcinomas were stained with the monoclonal antibody DO-7. 95 patients (39%) showed a high level of immunoreactivity (≥20% of cell nuclei staining positively), suggesting the presence of a mutation in the *TP53* coding sequence. Overexpression of the p53 protein correlated significantly with stage of disease ($P = 0.01$), the presence of distant metastases ($P = 0.04$) and with the intestinal type of cancer ($P = 0.04$). No correlation between p53 overexpression and age, gender or the presence of the lymph node metastases was found. In univariate analysis, p53 immunoreactivity correlated significantly with survival ($P = 0.0005$). The median survival in the p53 high-level group was 19 months compared with 65 months in the p53 low-level group. In multivariate analyses, stage of disease and the presence of distant metastases emerged as independent prognostic factors, whereas p53 immunoreactivity did not ($P = 0.08$). The present results indicate that overexpression of the p53 protein is not an independent prognostic factor in patients with gastric cancer.

Key words: gastric cancer, prognosis, p53, immunoreactivity, tumour suppressor gene protein
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INTRODUCTION

THE NUCLEAR phosphoprotein p53 was discovered in 1979 as a result of its binding to the large T antigen of the DNA tumour virus SV40 [1]. The *TP53* gene was considered a dominant oncogene because of its ability to co-operate with other dominant oncogenes such as RAS [2]. Subsequent studies have indicated that the *TP53* gene, in fact, is a tumour suppressor gene [3, 4], and changes in tumour suppressor genes have been suggested to be even more important than oncogenes in neoplastic transformation of epithelial cells [5]. Abnormalities of *TP53* have been found to occur frequently in a wide range of tumours and appear to be the most common molecular disorders occurring in human cancer [6–8].

The normal function of the p53 protein is apparently to detect and respond to DNA damage. p53 temporarily arrests the progression of the cell cycle in the presence of DNA damage, thus facilitating repair [9, 10]. The p53 protein also suppresses tumour growth by induction of apoptosis [11]. These functions are usually lost when a mutation has occurred in the short arm of chromosome 17, where the *TP53* gene is located [12]. The mutant p53 gene product has a prolonged half life and is, therefore, detectable by immunohistochemical methods. Accordingly, overexpression of the protein is considered to be an indicator of mutation [7, 13].

A review of the literature showed that some p53 abnormality could be demonstrated in nearly 40% of malignant tumours, most frequently in malignant melanomas (88%), and in testicular (87%) and colorectal cancer (58%) [14]. Overexpression of p53-mediated protein in gastric cancer has been demonstrated in 30–57% of the tumours [15, 16].

Despite the well-documented evidence of p53 overexpression in many types of cancer, reports concerning p53 overexpression and prognosis have been controversial. In some studies, no correlation has been found between p53 expression and clinicopathological variables related to biological aggressiveness [15, 17], while other studies have demonstrated a strong association between p53 expression and lymph node metastases [18–20].

The purpose of this study was to evaluate the prognostic significance of the immunohistochemical expression of p53-mediated protein in patients with gastric cancer, and to com-

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pare the expression with stage and other traditional indicators of prognosis.

PATIENTS AND METHODS

Patients

242 patients with gastric cancer treated at the Fourth Department of Surgery, University of Helsinki, Finland, between 1971 and 1993 were studied. 97 patients (40%) were treated with total gastrectomy, 43 (18%) with subtotal gastrectomy, and 72 patients (30%) underwent a gastric resection. 49 of the resection patients (68%) were treated with curative intent. 30 patients (12%) underwent only palliative procedures or explorative laparotomy. Thus, 189 patients in this study (78%) were operated on with the intent to cure.

The median age of the 118 males was 67 years, and that of the 124 females 68 years (range 26–90) at the time of operation. None of the patients received pre- or postoperative chemotherapy. Lymph node metastases were found at surgery in 101 patients, and distant metastases in 30 patients. The patients were classified according to the TNM stage of the tumours (UICC, 1987). There were 74 patients with stage I, 59 with stage II, 73 with stage III and 36 patients with stage IV cancer.

Median time of follow-up of patients was 13 years (range 2–23). 5 patients who died within 30 days of operation were excluded, leaving 237 patients for final analysis. Survival data were available on all patients and obtained from patient records, the Finnish Cancer Registry and the Population Registry.

Specimens

Tissue samples taken at operation were fixed in 4% buffered formaldehyde for 12–48 h, processed and embedded in paraffin and stored in the files of the Department of Pathology, University of Helsinki. Haematoxylin–eosin stainings of all samples were reviewed by one pathologist and the most representative samples were chosen for further analysis. The histological typing of the carcinomas was carried out according to the Laurén classification [21]. One hundred and twenty-seven tumours were diffuse and 115 were intestinal.

Immunohistochemical staining

Four-micrometre thick sections of the specimens were mounted on 3-aminopropyl-triethoxy-silane (APES) (Sigma, St Louis, Missouri, U.S.A.) coated slides and dried for 12–24 h at 37°C. The sections were deparaffinised in xylene and rehydrated through graded concentrations of ethanol to distilled water and processed in a microwave oven [22]. This procedure of antigen retrieval was found to be essential, since enzyme pretreatment, or no pretreatment at all, resulted in only weak staining with the p53 antibody.

Immunohistochemistry was performed using the avidin–biotin complex (ABC) immunoperoxidase technique applying a commercial Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, California, U.S.A.). Suppressor serum was applied for 15 min followed by overnight incubation with the monoclonal antibody DO-7 (Dako, Glostrup, Denmark), which recognises both mutant and wild-type p53 protein, diluted 1:300. The sections were incubated in the biotinylated second antibody and the peroxidase-labelled ABC for 30 min each.

All dilutions were made in phosphate-buffered saline (PBS) (pH 7.2) and all incubations in the ABC method were conducted in humidity chambers at room temperature. Between each step in the staining procedure (except before incubation with the primary antibody), the slides were rinsed in three changes of PBS.

The peroxidase staining was visualised with a 3-amino-9-ethyl-carbazole (Sigma, No A-5754) solution (0.2 mg/ml in 0.05 M acetate buffer containing 0.03% perhydrol, pH 5.0) at room temperature for 15 min. Finally, the sections were lightly counterstained in Mayer's haematoxylin and mounted in aqueous mounting medium (Aquamount, BDH, Poole, U.K.).

Sections of p53 positive breast carcinomas were included in every batch as positive controls.

Interpretation of immunohistochemical stainings

The staining results were interpreted independently by one pathologist unaware of the clinical outcome. The level of immunoreactivity was expressed as the percentage of p53-positive cancer cell nuclei. When samples from more than one site of the tumour were available, the mean percentage of positive nuclei was used. For analysis, tumours were classified into p53 immunoreactivity categories of low-level (negative or $<20\%$ positive nuclei) and high-level ($\geq 20\%$ positive nuclei). In multivariate analysis, the percentage of p53 positive cells was also entered as a continuous variable.

Statistical analysis

The χ^2 test was used to test for association between factors. Life-tables were calculated according to Kaplan–Meier. Deaths were deaths due to gastric cancer. Deaths due to other causes were treated by censoring. The statistical significance of the difference in survival of the groups was calculated using the log rank test. Multivariate survival analysis was conducted with the Cox proportional hazards model [23], entering the following covariates: p53 immunoreactivity level ($<20\% = 0$, $\geq 20\% = 1$, age (<67 years $= 0$, ≥ 67 years $= 1$), gender (male = 0, female = 1), stage group (stage I = 1, stage II = 2, stage III = 3, stage IV = 4), histological type according to Laurén (diffuse type = 0, intestinal type = 1), lymph node metastases (no = 0, yes = 1), distant metastases (MO = 0, M1 = 1). Separate Cox analysis was also performed entering the fraction of p53 positive cell nuclei as a continuous variable instead of dichotomising at 20%. Covariates were selected in a stepwise manner (backward to forward), using the maximum likelihood ratio. A P value of 0.05 was adopted as the limit for inclusion of a covariate.

RESULTS

The percentage of tumour cells showing p53 immunoreactivity varied from 0 to 90% (Figure 1, Table 1). Faint cytoplasmic staining without nuclear staining was considered negative. A high level of p53 immunoreactivity ($\geq 20\%$ positive nuclei), was seen in 95 of 242 patients (39%), indicating the presence of a *TP53* mutation. 53 (46%) of 115 patients with Laurén's intestinal type and 42 (33%) of 127 patients with the diffuse type gastric cancer had positive nuclear stainings ($P = 0.04$). There was a significant association between p53 immunoreactivity and stage of disease ($P = 0.01$), and the presence of distant metastases ($P = 0.04$). No association was seen between the level of p53 immunoreactivity and age of the patient, gender or presence of lymph node metastases (Table 2).

The survival time was significantly longer in patients with

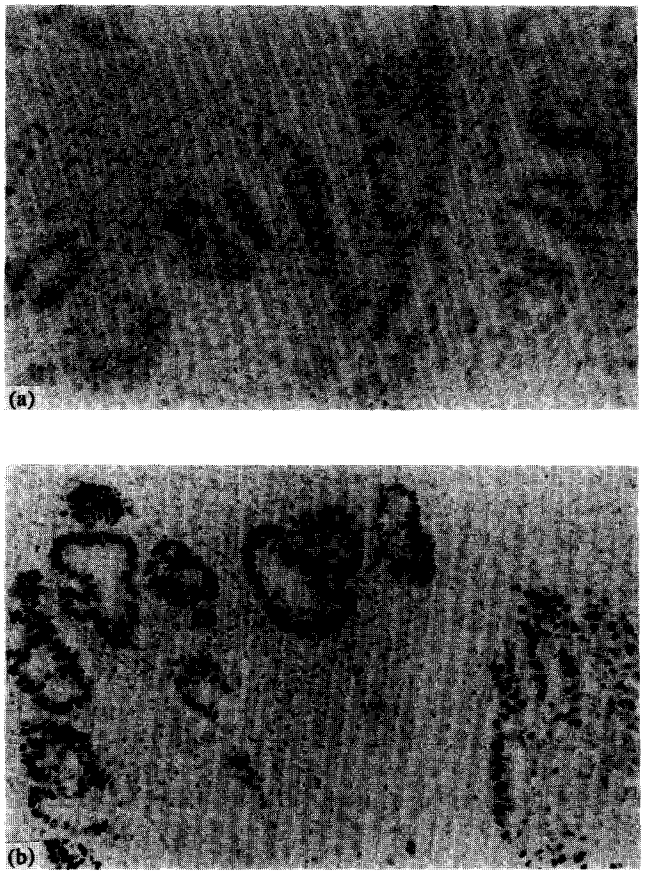

Figure 1. Immunohistochemical staining of p53 protein using monoclonal antibody DO-7. (a) Gastric adenocarcinoma with p53 immunoreactivity in only a few tumour cell nuclei. (b) Gastric adenocarcinoma with p53 immunoreactivity in the majority of cancer cell nuclei.

Table 1. Fraction of p53-positive tumour cell nuclei in 242 specimens of gastric adenocarcinoma

	p53 positivity (%)					
	0	1–19	20–39	40–59	60–79	80–100
Number of specimens	132	15	16	9	35	35

Table 2. Patients with gastric cancer showing a high level of p53 immunoreactivity (≥20% positive nuclei) according to pre-operative characteristics

Clinicopathological variable	Number of patients	Number of p53 positive patients (%)	χ^2	*P*
Age (years)				
<67	120	43 (36%)	1.17	0.28
≥67	122	52 (43%)		
Gender				
Female	124	43 (35%)	2.24	0.13
Male	118	52 (44%)		
TNM stage				
I	74	19 (26%)		
II	59	22 (37%)	10.89	0.01
III	73	35 (48%)		
IV	36	19 (53%)		
Laurén classification				
Intestinal type	115	53 (46%)	4.29	0.04
Diffuse type	127	42 (33%)		
Lymph node metastases				
Yes	101	45 (45%)	2.04	0.15
No	141	50 (35%)		
Distant metastases				
Yes	30	17 (57%)	4.35	0.04
No	212	78 (37%)		

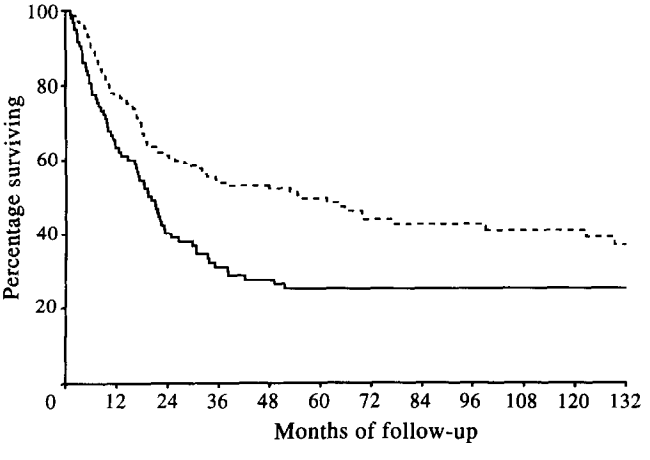

Figure 2. Kaplan–Meier life-tables for patients with gastric cancer with high (——) versus low (– –) levels of p53 immunoreactivity in tumour cell nuclei. The difference in survival between 142 patients with low levels (<20% of tumour cells positive) and 95 patients with high levels (≥20% of cells positive) was significant ($P = 0.0005$).

<20% p53-positive cells in their tumours than in those with ≥20% positive cells ($P = 0.0005$) (Figure 2). The median survival time of patients with high nuclear p53 immunoreactivity (≥20%) was 19 months compared with 65 months in patients with a low staining level. The 5-year survival rates were 25 and 50%, respectively. TNM stage showed the strongest association with survival in univariate analysis, followed by distant metastases, lymph node metastases and p53 immunoreactivity. Age, gender and histological type (Laurén) did not correlate significantly with survival (Table 3).

When the patients were stratified according to stage, there was no significant difference in survival between patients with positive and negative p53 immunostainings (Figure 3a–d). In stage II cancer, the median survival time was 49 months and 22 months (Figure 3b), in stage III cancer it was 17 months in both groups (Figure 3c), and in stage IV cancer 7 months and 4 months, respectively (Figure 3d).

In multivariate analysis, stage was the strongest independent prognostic factor, followed by the presence of distant metastases (Table 4). The level of p53 immunoreactivity (cut-off point 20% positive nuclei), lymph node status, histological

Table 3. Univariate analysis of the relationship between preoperative characteristics and survival in 237 patients with gastric cancer

Clinicopathological variable	Number of patients	Median survival in months	χ^2	*P*
p53 immunoreactivity				
High level (≥20%)	95	19	12.30	0.0005
None or low level (<20%)	142	65		
Age (years)				
<67	118	34	1.62	NS
≥67	119	22		
Gender				
Female	123	34	0.40	NS
Male	114	23		
TNM stage				
I	73	63		
II	58	49	170.48	<0.0001
III	71	17		
IV	35	6		
Laurén classification				
Intestinal type	112	27	0.07	NS
Diffuse type	125	31		
Lymph node metastases				
Yes	99	11	86.26	<0.0001
No	138	54		
Distant metastases				
Yes	29	6	124.97	<0.0001
No	208	42		

The p53 immunoreactivity categories were low- (<20% positive nuclei) or high-level (≥20% positive nuclei). Chi-square and corresponding *P* values were calculated by the log rank test. NS, non-significant.

type (Laurén), age and gender did not predict prognosis independently at a significance level of 5%. Entering the fraction of positive cell nuclei as a continuous variable instead of dichotomising at 20% did not alter the results significantly.

DISCUSSION

A high level of p53 immunoreactivity in cell nuclei of gastric adenocarcinomas (≥20%) was observed in 95 (39%) of the 242 cases included in this study. These findings agree with previous reports, where a positive nuclear staining has been found in 30–57% of gastric adenocarcinomas [15–18, 20, 24, 25]. In our study, the p53 staining was positive more often in the intestinal type than in the diffuse type of gastric cancer. This is consistent with the results of Martin and associates [15] and Fukunaga and associates [25] but in conflict with the results of Joypaul and associates [17] and Starzynska and associates [18] who found no such correlation. These differences might be explained by differences in patient materials or differences in interpretations of histological findings.

A mutation in the gene coding for the *TP53* protein and subsequent abnormal p53 expression has been found to correlate with poor prognosis in many human cancers. To our knowledge, only three previous studies have correlated p53 expression with prognosis in patients with gastric cancer. Starzynska and colleagues [18] found a correlation between p53 overexpression and poor overall survival, but survival was not correlated to stage of disease or to other known prognostic factors. Martin and colleagues [15] and Joypaul and colleagues [17] found p53 overexpression in gastric cancer to be an independent prognostic factor in multivariate analysis, which is in conflict with our findings. Although we found p53 overexpression to be significantly correlated to poor overall survival, it did not emerge as an independent prognostic factor in a Cox proportional hazards model. These differences between our findings and those of previous studies may be due to differences in the number of patients examined. Our study included 237 patients for survival analyses, whereas the previously mentioned studies [15, 17, 18] were performed on a much smaller number of patients. We found a stronger association between p53 expression and stage, which partly explains the differences in observed prognostic value of p53 overexpression. Moreover, differences in affinities between antibodies against the same antigen and differences in immunohistochemical staining procedures might explain some of the differences.

Intratumoral variability in p53 expression has been described in most reports, and p53 immunoreactivity varied within the same tumour in our series as well. Nuclear staining of the majority of tumour cells in the absence of a positive reaction in surrounding normal tissue is the most commonly observed pattern in missense *TP53* mutations [13], whereas p53 expression in only a small fraction of cell nuclei correlates poorly with a mutation of the *TP53* gene [14, 26]. No molecular analyses of the samples were performed in this study. However, the immunohistochemical expression of p53 correlates well with molecular analyses [27].

In many studies showing p53 to be of prognostic value, tumours have been divided into those with ≥20% positive cell nuclei, and those with low or no reactivity (<20%) in cell nuclei [28–32]. We used the same cut-off value in this study, but we also entered the fraction of p53 positive cells as a continuous variable in multivariate analysis. This, however, did not alter the results. Although the significance level somewhat improved, statistical significance was not reached.

Conflicting results concerning p53 nuclear immunoreactivity and prognosis have been reported in other cancer forms as well. Several studies on colorectal, cervical and lung cancers claim that p53 immunoreactivity is not a prognostic factor [33–36], while other studies claim p53 to be of prognostic value [37–40]. The reason for these variations in results is unknown. One explanation for the lack of prognostic significance of p53 evaluated from immunohistochemical stainings might be the fact that the concordance between a *TP53* gene mutation and the accumulation of p53 protein is not perfect. It is possible that other molecular alterations affecting the synthesis and stability of p53 may have occurred in tumours with a high level of p53 immunoreactivity [13]. Conversely, absence of p53 staining is not synonymous with normal p53 function. In frame-shift or nonsense mutations, *TP53* is generally undetectable by immunohistochemical techniques because the resultant protein is truncated, unstable or absent [8].

In conclusion, we found p53 immunoreactivity to be significantly correlated to overall 5-year survival, to stage of disease, to the presence of distant metastases and to the

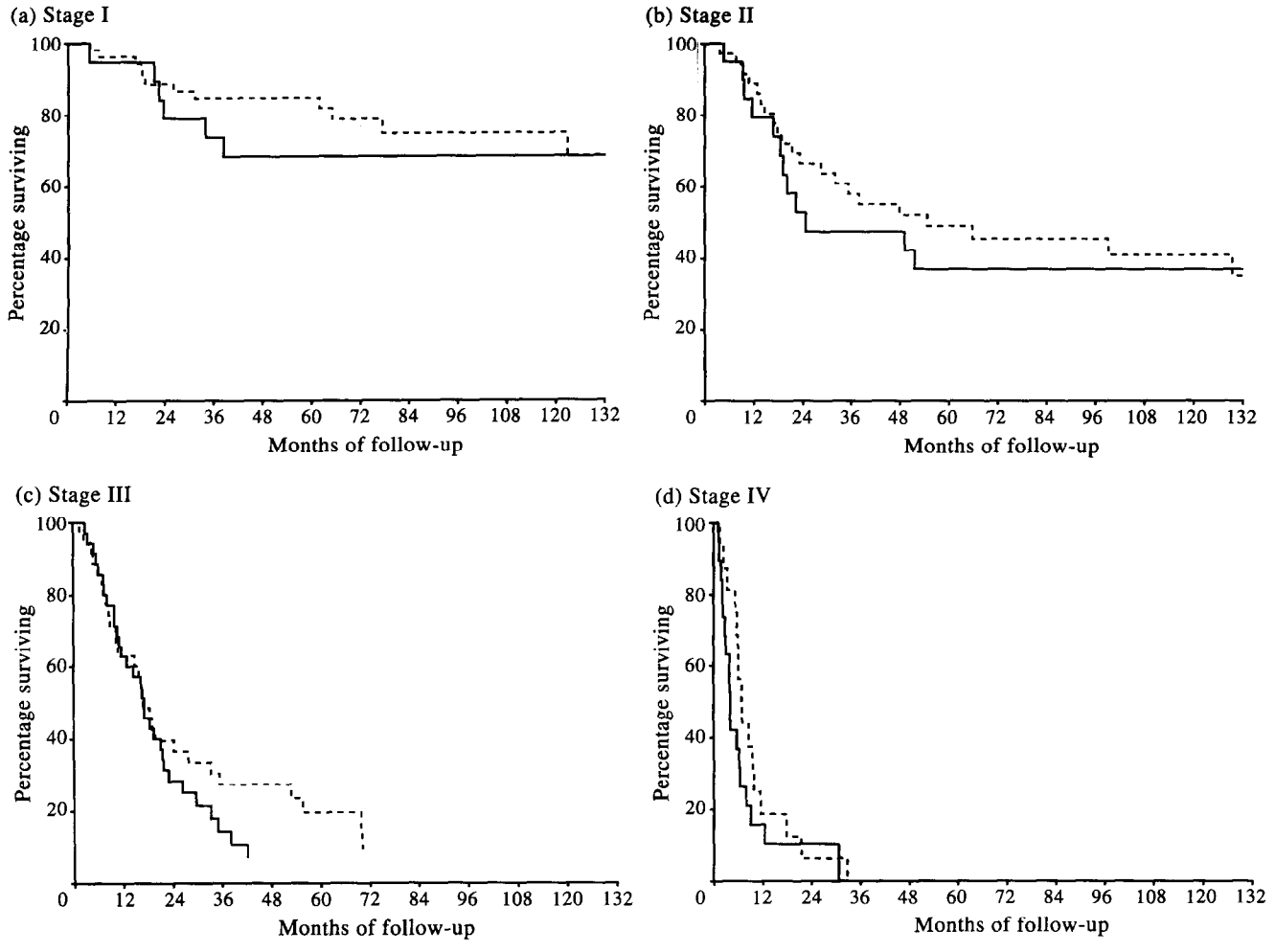

Figure 3. Kaplan–Meier life-tables for patients with gastric cancer with high (——) versus low (– –) levels of p53 immunoreactivity in tumour cell nuclei in (a) stage I cancers, (b) stage II cancers, (c) stage III cancers, (d) stage IV cancers. The differences in survival were non-significant.

Table 4. Stepwise multivariate analysis (Cox proportional hazards model, backward to forward, P to remove 0.05) of prognostic covariates of survival in 237 patients with gastric cancer

Covariate	*P*	RH	CI (95%)
TNM stage	<0.0001	2.48	1.99–3.09
Distant metastases	0.04	1.75	1.03–2.98
p53	NS (0.08)	1.35	0.97–1.88
Nodal metastases	NS		
Laurén classification	NS		
Age	NS		
Gender	NS		

Stage was entered as an ordinal variable (stage I = 1, stage II = 2, stage III = 3, stage IV = 4). Distant metastases were entered as ordinal variables (MO = 0, M1 = 1). p53 immunoreactivity categories were entered as low-level (<20% positive nuclei) = 0, and high-level (≥20% positive nuclei) = 1. Lymphatic nodal metastases were entered as no = 0, yes = 1. Histological type according to Laurén was entered as diffuse type = 0, intestinal type = 1. Age was entered as <67 years = 0, ≥67 years = 1. Gender was entered as male = 0, female = 1. RH, relative hazard; CI (95%), confidence interval at 95% level; NS, non-significant.

intestinal type of gastric cancer. Both in univariate and multivariate analysis, stage of disease was the strongest predictor of poor survival followed by the presence of distant metastases. High p53 immunoreactivity was also significantly correlated with poor survival in univariate, but not in multivariate analysis. According to the present results, p53 overexpression is not an independent prognostic factor in patients with gastric cancer.

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