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RESEARCH

Prognostic Significance of p53 Protein Expression in Early Gastric Cancer

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Abstract Mutations of the p53 tumor suppressor gene have been associated with abnormalities in cell cycle regulation, DNA repair and synthesis, apoptosis, and it has been implicated in the prognosis of advanced gastric cancer. The aim of this study was to evaluate the occurrence of p53 gene mutation and its possible prognostic implications in early gastric cancer. In a retrospective study, we studied 80 patients with early gastric cancer treated surgically between 1982 and 2001. Mutation of p53 gene was investigated in surgical gastric specimens by immunohistochemistry, and results were analyzed in relation to gender, age, macroscopic appearance, size and location of tumor, presence of lymph nodes, Lauren's histological type, degree of differentiation, and the 5-year survival. The expression of p53 was more frequent among the intestinal type (p = 0.003), the differentiated (p = 0.007), and the macroscopically elevated tumors (p = 0.038). Nevertheless, the isolated expression of p53 was not associated with the 5-year survival, or with the frequency of lymph node involvement. The degree of differentiation was detected as an independent factor related to the outcome of patients (0.044). Significantly shorter survival time was found in p53-negative compared with p53-positive patients, when considering the degree of differentiation of tumors, as assessed by Cox regression analysis (0.049). The association of p53 with the intestinal type, the degree of differentiation and morphological characteristics, may reflect the involvement of chronic inflammatory process underlying early gastric cancer. In this population sample, the expression of p53 alone has no prognostic value for early gastric cancer. However, the significant difference in p53 expression between subgroups of degree of differentiation of tumors can influence post-operative outcome of patients and may be related to possible distinct etiopathogenic subtypes.

Keywords Early gastric cancer · p53 · Prognosis

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Introduction

Gastric carcinoma is one of the most common visceral cancers in the world [1, 2]. Despite recent advances in the understanding of the biology and development of gastric cancer, therapeutic effectiveness has been limited and the prognosis for patients is still poor even in developed countries [3, 4]. Vascular and lymp node invasion are usually regarded as indicators of recurrence and poor

outcome commonly seen in advanced gastric cancer [5, 6]. In contrast, early gastric cancer defined as a tumor confined to the mucosa or submucosa is usually associated with a better prognosis [7].

Potential molecular markers for gastric cancer have been pursued and insights into genetic alterations have evolved considerably in the recent years. Currently, it is thought that gastric carcinogenesis involves multiple genetic alterations in a gradual process, and it seems that inadequate functioning of regulatory mechanisms of apoptosis could result in tumorigenesis [8, 9]. The p53 tumor suppressor gene appears to play a pivotal role in human carcinogenesis [10] and *p53* mutations have been frequently reported in human cancer [11, 12]. The p53 gene encodes a nuclear phosphoprotein, which functions as a transcription factor implicated in the regulation of the cell cycle, synthesis and in DNA repair and apoptosis [13]. Mutations of the p53 gene consequently leading to inactivation of p53 protein tumor-suppressor activity appear to constitute one of the most common molecular steps in the development of cancer [14, 15]. There are evidence indicating the existence of association between point missense mutations in the *p53* gene and p53 protein overexpression in tumors [15], which is believed to result from a prolonged half-life of the mutant protein compared with the wild-type p53 [11].

Different studies indicate the association of p53-overexpression with gastric cancer and the resulting reduced survival time for the tumor [16–18]. Other studies fail to support the significance of p53 expression in the outcome of gastric cancer [19, 20]. Of note, contradicting data may derive from differences in patient populations, but also in immunohistochemical methods employed for the detection of p53.

In advanced gastric cancer, it has been suggested that tumors containing the wild-type p53 are more sensitive to chemotherapeutic agents [21]. On the other hand, in early gastric cancer p53 has been reported to be associated with the presence of metastases to lymph nodes, with consequent impact on prognosis [22]. Overall, the clinical significance and potential applications of p53 in gastric cancer remain controversial. In the present study, we investigated the possible association of p53 protein expression with clinical and pathological variables and its role in the post-operative outcome of patients with early gastric cancer.

Materials and Methods

Patients and Samples

Eighty consecutive patients with the diagnosis of early gastric cancer were submitted to potentially curative surgery at the Department of Surgical Oncology, National Institute of Cancer, during the period from 1982 to 2001. Patients with early gastric cancer consisted of 42 men (52.5%), and 38 women (47.5%), with a mean age of 60 years (std. deviation 14.2 years) and median of 61 years (range 33–86 years). The surgically resected specimens used for this study consisted of 37 cases in which tumors were confined to the mucosa, and 43 cases in which tumors had reached the submucosa. In regard to follow-up, patients who died within 1 month after surgery, or died of unrelated causes within 3 months were excluded from this study. The histological types of tumors were reviewed and classified as intestinal or diffuse according to Lauren [23]. None of the patients had received either chemotherapy or radiation therapy before surgery.

Formalin-fixed paraffin-embedded blocks from surgical specimens of primary tumors were retrieved from pathological archives. The most representative slide was selected from each block obtained after careful review of all slides from each case by the same pathologist (I.M.). Selected tissue samples were subsequentely cut into 3 μm sections at the maximum cross-section of the tumor, onto slides pretreated with poly-L-lysine (Sigma Chemical Co., St Louis, MO, USA), and processed for the imunohistochemical study.

The study protocol was approved by the Ethical Committee of the National Institute of Cancer, Rio de Janeiro, and informed consent was obtained from all patients.

Immunohistochemistry

For this set of experiments, paraffin sections were used to characterize the expression of p53 in early gastric cancer, which was performed by using the indirect immunoperoxidase technique. Immunohistochemical staining for p53 was carried out using a monoclonal mouse anti-human p53 antibody diluted 1:50 (DO-7; DAKO) as primary antibody.

Briefly, paraffin-embedded gastric samples were dewaxed in xylene twice for 5 min each time and then rehydrated in graded ethanol (100–70%) three times, followed by rehydration in phosphate buffered saline (PBS), and antigen retrieval by pressure cooking. For antigen retrieval, sections were immersed in a 10 mM sodium citrate buffer (pH 6.0), and heated in a pressure cooker two times for 3 min each at a 10-min interval.

Slides were then immersed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After being rinsed in PBS containing 0.5% Tween 20 for 10 min, tissue sections were incubated with non-immune horse serum for 30 min and, subsequently, with the anti-p53 monoclonal antibody in a humidified chamber overnight, at 4oC. Two sections from each sample were incubated with either PBS alone or mouse monoclonal IgG1 (concentration-

matched) (Dako A/S, Glostrup, Denmark) and served as negative controls. Positive controls were obtained from known positive cases of colon cancer. After being rinsed in PBS for 10 min, all tissue sections were incubated for 30 min with a goat anti-mouse peroxidase conjugate (1:200) (Zymed Laboratories, Inc., San Francisco, CA, USA). Additional rinsing was followed by development with a solution containing hydrogen peroxide and diaminobenzidine, and hematoxylin was used for counterstaining. Slides were then dehydrated and mounted in histological mounting medium.

Assessment of p53 Expression

A semi-quantitative analysis of tissue sections (under light microscopy at × 400 magnification) was carried out by using a computer-assisted image analyser (Image-Pro Plus Version 4.1 for Windows, Media Cybernetics, LP, Silver Spring, MD, USA). A distinct nuclear immunoreactivity for p53 was recorded as positive, and the nuclear staining pattern was usually diffuse. For tumors that showed heterogeneous staining, the predominant pattern was taken into account for scoring. Cases with less than 10% positively stained cancer cells nuclei were defined as negative, otherwise they were defined as positive [17].

Statistical Analysis

Statistical analysis was performed using the statistical software SPSS for Windows (Version 10.0.1, SPSS Inc., 1989–1999, USA). The Chi-square test was used to analyze the expression of p53 in the context of various clinical and pathological variables. Estimation of overall and disease-free survival rates was calculated using the Kaplan-Meier method, and differences between curves were assessed with the log-rank test. Simultaneous multivariate adjustment of all covariates was performed using the Cox proportional hazards regression analysis with the forward stepwise model, to evaluate the independent importance of p53 for survival after resection. Combinations of potentially confounding variables in regard to p53 alterations were tested. The level of significance was set at $p < 0.05$.

Results

Of the 80 samples of early gastric cancer studied, 22 (27.5%) overexpressed the p53 protein. The p53 staining was characteristically nuclear in tumoral tissue and was completely absent in the normal gastric mucosa in all cases, as shown in Fig. 1.

Clinicopathological Features

The clinicopathological data of both p53-positive and p53-negative patients are shown in Table 1.

The expression of p53 was significantly more frequent in intestinal than in diffuse Lauren's histological type ($p = 0.003$). The degree of differentiation, was also significantly associated with p53 expression which was found more often in cases presenting grades I and II (differentiated),

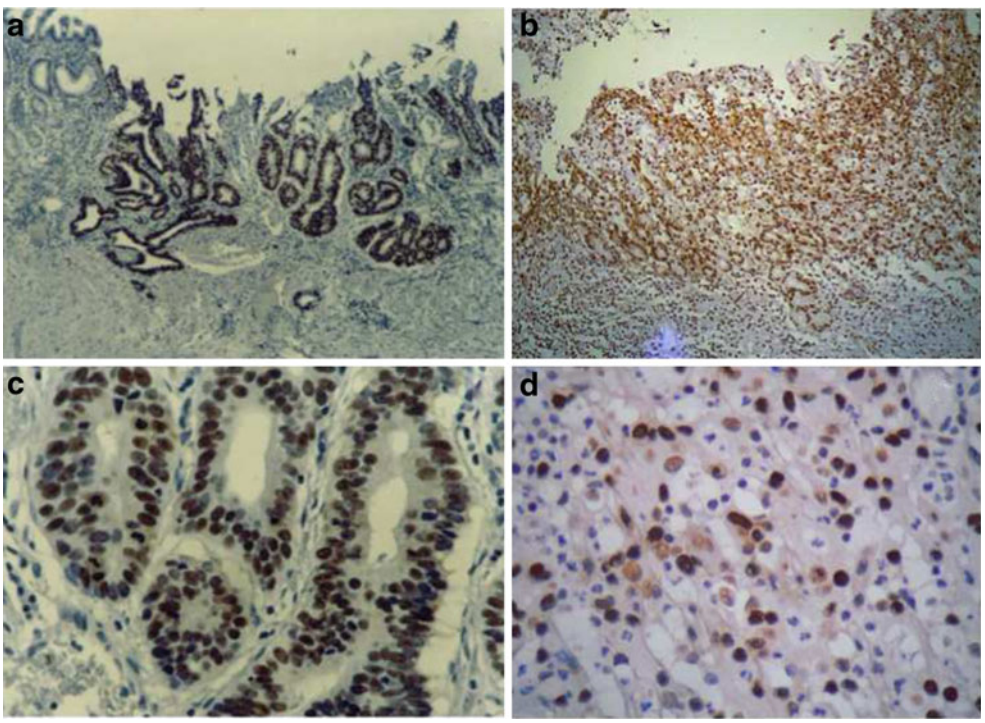

Fig. 1 Immunohistochemical detection of p53 protein using anti-p53 monoclonal antibody in paraffin sections of early gastric adenocarcinoma. Slides show the tubular pattern of grade I (*A*, *B*) and grade III (*C*, *D*) early gastric carcinomas, at × 100 magnification, and the characteristic nuclear staining at × 400 magnification, respectively

Table 1 Clinicopathological features and p53 status in patients with early gastric cancer

Variable	p53 negative (n = 58)	p53 positive (n = 22)	p-value
Age (years)	64.3 ± 13.6	58.4 ± 14.2	0.890
Gender			
male	29	9	0.467
Female	29	13	
Tumor diameter (cm)	4.88 ± 2.44	3.58 ± 1.51	0.095
Tumor location			
Upper	11	7	0.289
Middle	19	4	
Lower	28	11	
Macroscopic type			
Elevated	7	7	0.038
Depressed	51	15	
Differentiation			
Grade I + II	25	18	0.004
Grade III	33	4	
Histological type			
Intestinal	32	18	0.003
Diffuse	26	4	
Tumor depth			
Mucosa	38	16	0.539
Submucosa	20	6	
Lymph node			
Positive	11	4	0.936
Negative	47	18	

NS no significant difference; figures in parentheses are percentages; *a* mean ± standard deviation; b unknown and specific cases were excluded from statistical analysis.

rather than grade III (poorly differentiated) tumor type (p = 0.007). In respect of the macroscopic appearance of tumors, overexpression of p53 was found more frequently in the elevated compared to the depressed forms (p = 0.038). The expression of p53 was not significantly associated with age, gender, tumor location, tumor diameter, depth of tumor invasion, and lymph node involvement.

Survival Analysis

The overall 5-year post-operative survival rate was 86.2% for patients with p53-negative tumors and 86.3% for p53-positive tumors (p = 0.985). Deaths which were not attributed to gastric cancer were regarded as censored data in the statistical analysis. Of all the other clinicopathological factors analyzed in this study, only the degree of differentiation was found to significantly affect the survival of patients (p = 0.044). The survival curve for patients with p53-negative or p53-positive early gastric cancers was determined for each grades I and II (differentiated) and grade III (undifferentiated) type of tumor group. Significantly shorter survival time was noted in p53-negative patients compared with p53-positive patients, when considering the degree of tumor differentiation (Fig. 2).

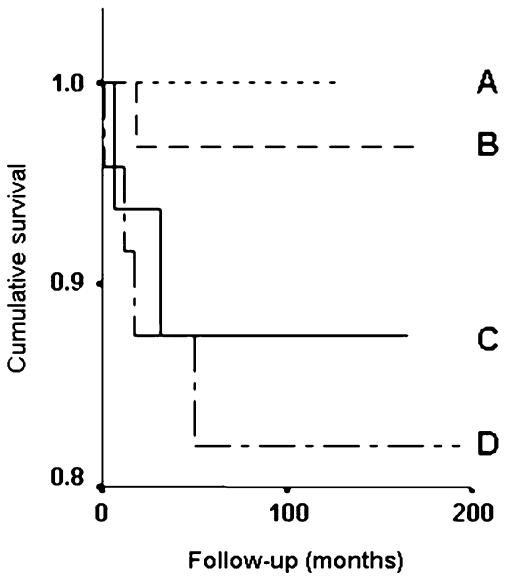

Fig. 2 Kaplan-Meier survival curve for patients after surgical resection of primary early gastric adenocarcinoma categorized according to p53 status and the degree of tumor differentiation (**a**, p53-positive/grade III; **b** p53-negative/grade III; **c** p53-positive/grades I+II; **d** p53-negative/grades I+II). Significantly improved overall survival was seen for patients with p53 overexpression, and for grade III tumors (p = 0.049)

Multivariate Analysis of p53 Protein Expression

The relationship between p53 expression and other possibly prognostic variables, such as age, sex, tumor size, lymph node involvement, was studied using the Cox multivariate analysis (Table 1). Next, we sought to determine survival rates for patients in regard to the macroscopic type of tumor, the degree of differentiation, and the Lauren's histologic type, identified as factors related to p53 expression. Variables were eliminated from the model stepwise in a backward fashion being reincluded whenever $p < 0.05$. With this analysis we identified the degree of differentiation as the only significant variable, with a relative risk of 4.63 (95% confidence interval, 0.93–23.02), consisting of an independent factor related to the outcome of patients and for p53 expression.

Discussion

In order to explore potential clinical applications for the p53 tumor suppressor gene, this retrospective study was carried out to characterize p53 alterations in a well-defined series of surgically resected early gastric adenocarcinomas. We studied 80 early gastric tumors, documenting p53-positive staining in 27.5% of cases. In regard to clinico-pathological findings, p53-positive staining was significantly associated with the macroscopically elevated tumors, the Lauren's intestinal histological type, and grades I and II tumors. The 5-year post-operative survival analysis showed that p53 alone does not represent a prognostic factor. However, with a multivariate analysis, the histologic degree of differentiation of tumors was found to represent an independent factor related to the outcome of patients and also for p53 expression.

The loss of p53 function is thought to play a critical role in the development of tumors since point mutations of the p53 tumor suppressor gene constitute one of the most frequent molecular alterations implicated in human malignancies [12, 24, 25]. Loss of p53 function would change the phenotype of neoplastic cells, making them more sensitive to DNA damage, and accelerating the process of tumorigenesis [26, 27]. In gastric carcinogenesis, p53 mutation is regarded as a common event, appearing from the early stage of gastric adenocarcinoma with its specific mutation spectrum, and also in lesions regarded as tumor precursors [28, 29].

In normal conditions, the p53 gene is believed to function as a transcription factor having a variety of biologic actions, including the regulation of cell cycle, apoptosis, and maintenance of genomic integrity [10]. In virtue of its rapid turnover, the phosphoprotein encoded by the p53 gene usually does not accumulate in normal cells, but following diverse cellular signals, the p53 protein stabilizes to activate downstream targets [13, 30]. Here, we utilized immunohistochemistry to assess possible associations between the expression of p53 with diverse clinical and pathological variables, and survival, in surgical samples from 80 patients with the diagnosis of the early type of gastric cancer. The antibody DO-7, utilized in this study, recognizes both the wild-type and mutant forms of p53 [31]. The extended half-life of the mutated p53 protein makes it more likely to be detected by immunohistochemistry than the wild-type protein [32, 33].

In this study, no p53 staining was observed in any normal gastric mucosa adjacent to the tumor tissue and the short half-life of the wild-type protein supports the suggestion that the immunoreactivity to p53 may reflect the presence of mutant forms. However, in contrast to previous studies [17, 20], we did not find any relationship between p53 expression and a possible more aggressive biological behaviour of tumors and enhanced proliferating activity of cancer cells. Differently from studies aiming at advanced gastric cancer, the p53 abnormal staining in this study was not related to the depth of tumor invasion, lymph node metastasis, and to survival of patients [34, 35, 36].

The occurrence of p53 mutation in our series was shown to be related to Lauren's histological type and the degree of differentiation, being more frequent in grade I and II tumors of the intestinal type. This is in accordance with a previous study on gastric cancer that reported the association of p53 mutation with the histological intestinal type [37]. In fact, it has also been proposed that p53 mutation could constitute a common genetic modification found in the progression of both histological types of tumor, possibly appearing at different time-points [38]. The eventual finding of p53 mutation in both histological types has been hypothesized as an early event in intestinal type and a later event in the diffuse type [39, 40].

Another possible explanation for the association between p53 and gastric tumorigenesis derives from the current knowledge on the developmental process of gastric cancer of the intestinal type. Gastric cancer of the intestinal type appears to result from a multi-step process, including atrophic gastritis, intestinal metaplasia, and dysplasia, all of them associated with the existence of a chronic inflammatory process [41]. In the context of chronic gastritis, *H. pylori* infection emerges as another factor capable of inducing the expression of the mutant-type p53 [42, 43], which was shown to be closely associated with the more severe atrophic and metaplastic changes [44], and also in gastric cancer [45].

The prognostic implications of p53 mutation in human malignacies remain controversial. While some investigators support the relationship of p53 with cancer prognosis [46, 47], others reported that p53 overexpression is actually not

related to the prognosis of neoplastic diseases, including gastric cancer [19, 20]. In this study, the overexpression of p53 showed no significant relationship with the outcome of patients. Indeed, it is possible that an association of p53 with cancer prognosis could eventually appear if longer follow-up periods have been applied, in particular for early gastric cancer patients, whose recurrence rates are already expected to be low. However, when analyzing p53 together with different variables in a model of multivariate analysis we demonstrated the association of p53 with the histologic degree of differentiation of tumors, which was shown to independently impact the outcome of patients. It is possible that p53 may play an importat role in the initiation of a variety of tumors, whereas the specific oncogene involved initially would be probably irrelevant once a tumor has evolved [48]. In addition, some reports support the suggestion that a combined assessment of expressions of p53 with other factors, such as cyclin E and vascular endothelial growth factor could help in the evaluation of tumor aggressiveness and prognosis for various cancers [49], and also with molecules such as HER2 and survivin, being significantly implicated in the prognosis of patients with breast [50] and gastric cancers (51), respectively.

In conclusion, in this study, the isolated expression of p53 has no prognostic impact in early gastric cancer. The association of p53 with the intestinal type, the degree of differentiation and morphological characteristics, may reflect the involvement of specific pathological processes underlying probable distinct subtypes of early gastric cancer. Further studies with more patients and a longer follow-up, and including different molecular biomarkers in addition to p53, will help in the understanding of disease pathogenesis and possibly in inidentifying prognostic factors to guide novel therapeutic approaches.

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