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Overexpression of p53 predicts shorter survival in diffuse type gastric cancer

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Background It has been suggested that p53 plays an important part in gastric carcinogenesis but the data remain inconclusive.
Methods Alteration of the tumour suppressor gene *p53* was prospectively investigated by immunohistochemistry in 168 primary gastric cancers.
Results Positive staining, indicative of gene mutations, was detected in 34 tumours (20·2 per cent). No correlation was observed between expression of p53 and various clinicopathological factors, including age, sex, tumour site, gross type, tumour size, depth of invasion, lymph node metastases, distant metastases, and tumour node metastasis stage. However, p53 overexpression was different between intestinal and diffuse type gastric cancer. Survival analysis revealed a significant survival disadvantage of p53 expression in diffuse type gastric cancer ($P = 0{\cdot}039$) but not in the intestinal type. Multivariate analysis of all 168 patients revealed that independent predictors of recurrent disease included age, invasion depth and nodal involvement but not p53 expression.
Conclusion The presence of p53 overexpression may identify a subset of more aggressive tumours with a poor prognosis in diffuse type gastric cancer.

It has been suggested that the most common genetic alternation in human cancers involves the loss of inhibitory function of the *p53* tumour suppressor gene product[1]. The *p53* gene mapped on chromosome 17p is an important negative regulator of normal cell growth and division. A common mechanism for this change appears to be the loss of one normal *p53* allele and point mutation of the remaining *p53* allele[2]. The resultant effect of these genetic alternations on intracellular p53 is a paradoxical increase in protein levels, since mutated p53 has a much longer half-life than normal p53. Accordingly, detectable levels of p53 protein product by immunohistochemistry suggest the existence of genetic alternations at this locus, since the very low steady-state levels of the normal protein (due to its rapid turnover) are usually invisible by this method[3]. Mutation of p53 and a raised protein level as determined by immunohistochemistry has been described in different types of human cancer[4–6].

Gastric cancer is a common disease worldwide and the third leading cause of cancer death in Taiwan[7]. Although aggressive surgical resection has improved the overall outcome of patients with this carcinoma, results of surgical resection for advanced cancer are still poor[8]. To search for predictors of disease survival and response to therapy is therefore mandatory. The molecular events leading to the development of gastric cancer are largely unknown, but there is now enough evidence to suggest that the functional inactivation of the *p53* gene through allelic loss and point mutation plays an important part[8–12]. Furthermore, several studies have shown recently that p53 mutations are likely to be associated with tumour metastases, stage and clinical outcome in breast cancer[13–16].

The aim of the present study was to determine whether p53 status in patients with gastric cancer patients who have undergone gastrectomy correlates with various clinicopathological characteristics and survival.

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Patients and methods

Patients

Between July 1992 and December 1994, 168 patients who had undergone gastric resection for primary gastric adenocarcinoma were studied. No patient had undergone previous chemotherapy or radiotherapy and all patients were followed clinically for a median of 4 years. All the clinicopathological data were collected prospectively and registered in a database. The operation was performed according to the guidelines of the Japanese Research Society for Gastric Cancer[17]. Radical lymph node dissection ($R_{2/3}$) was performed routinely. A detailed histopathological examination was performed to determine the depth of invasion on the gastric wall and the extent of metastases within regional lymph nodes. The clinical stage was determined by the tumour node metastasis (TNM) system[18]. The histological diagnosis was made according to the classification of Laurén[19].

Immunohistological staining

Cancer and normal mucosa samples were taken immediately after surgical excision and snap frozen, then stored at −70°C until use. For immunohistochemical demonstration of the p53 protein, sections of the freshly frozen tissues were cut at 6 μm and stained with specific monoclonal p53 antibody (Oncogene Science, New York, USA) by an avidin–biotin complex peroxidase complex method. Cryosat sections of 5–7 μm were cut and mounted on glass slides, then treated with 3 per cent hydrogen peroxide in methanol at room temperature for 30 min to block endogenous peroxidase. The tissue sections were then incubated for 20 min with normal non-immune goat serum from the same species as the secondary antibody. Excessive normal goat serum was blotted from the slides before incubation with the primary antibody. Sections were incubated with primary mouse monoclonal antibody against p53 gene product (Oncogene Science) at

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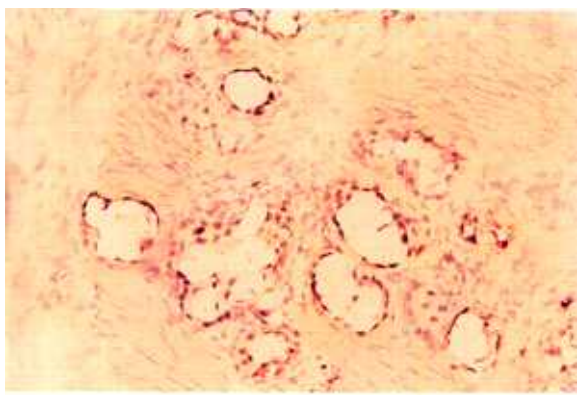
1:50 dilution overnight in a moist chamber at 4°C. Sequentially, the sections were incubated with biotinylated goat antimouse antibody for 30 min at room temperature and peroxidase-conjugated streptavidin was applied for 10 min at room temperature. Tissue sections were then stained with 0·05 per cent 3′,3 diaminobenzidine tetrachloride freshly prepared in 0·05 mol/l Tris-hydrochloride (pH 7·6) containing 0·01 per cent hydrogen peroxide and checked by light microscopy to determine the colour of chromogen. Finally, sections were counterstained with Mayer's haematoxylin, or methyl green, and then mounted. The reaction was considered as positive when a strong colouration of the cell nucleus was evident. Negative control sections were processed immunohistochemically without the primary antibody and positive control sections were from a breast cancer known to express high levels of p53.

Statistical analysis

Data were analysed using the χ^2 test with Yates' correction and Student's t test to evaluate the statistical significance of the relationship between staging and other histopathological factors. Cumulative survival rates were calculated using the Kaplan–Meier method. Statistical differences between two survival curves were evaluated using the generalized Wilcoxon test and savage test. Multivariate analysis, Cox model of proportional hazards regression, was used to incorporate all of the explanatory variables with the greatest prognostic value[20]. $P<0·05$ was considered to be statistically significant. The analysis was done using the Biomedical statistical package (BMDP Statistical Software, Los Angeles, California, USA).



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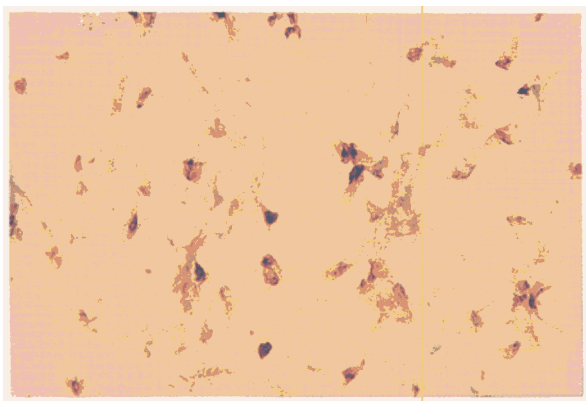

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Fig. 1 Immunohistochemical staining of p53 protein in gastric cancer using monoclonal antibody. **a** Intestinal type cancer; p53 protein expression is seen primarily on cell nucleus (original magnification ×200). **b** Diffuse type cancer; cell nucleus staining is exhibited (original magnification ×400)

Results

Overexpression of p53

Nuclear staining of tumour cells was considered a positive p53 overexpression (*Fig. 1*). Nuclear positivity was not seen in any of the ten specimens of normal gastric mucosa used as controls or in the non-cancerous mucosa adjacent to the carcinoma.

An overall positive staining was seen in 34 (20·2 per cent) of 168 gastric cancers. The clinicopathological data relating to the resected tumours in both groups are summarized in *Table 1*. There was no significant correlation between p53 protein expression and any of the clinicopathological variables, including age, sex, tumour location, gross type, histological type, depth of invasion, lymph node metastases, distant metastases and TNM stage. Although p53 expression tended to be associated

Table 1 Association of p53 overexpression with clinicopathological findings in 168 patients with resectable gastric cancer

Variable	No. of patients	p53 negative	p53 positive
Total no.			
Age (years)			
≤40	12		
>40	156		
Sex			
M	90		
F	78		
Location			
Upper	37		
Middle	35		
Lower	96		
Macroscopic type			
Early cancer	23		
Borrmann I	7		
Borrmann II	22		
Borrmann III	101		
Borrmann IV	15		
Size			
<4 cm	53		
4–8 cm	89		
>8 cm	26		
Depth of invasion			
T_1	23		
T_2	41		
T_3	90		
T_4	14		
Lymph node metastases			
N_0	55		
N_1	38		
N_2	49		
N_3	26		
Distant metastases			
Negative	149		
Positive	19		
Histology			
Intestinal	72		
Diffuse	83		
Mixed	13		
TNM stage			
	39		
II	26		
III	65		
IV	38		

Values in parentheses are percentages. TNM, tumour node metastasis. No correlation was observed between p53 expression and any of these variables

Table 2 Comparison of p53 overexpression between intestinal and diffuse type gastric adenocarcinoma

Intestinal type + diffuse type ($n = 155$)	Intestinal type ($n = 72$)	Diffuse type ($n = 83$)
T_1 3 of 23	3 of 12	0 of 11
T_2 6 of 38	5 of 20	1 of 18
T_3 21 of 80	10 of 36	11 of 45
T_4 2 of 14	0 of 4	2 of 10

with advanced rather than early cancer (21·4 *versus* 13 per cent) and intestinal than diffuse cancer (25 *versus* 17 per cent), this had no statistical significance. A significant difference was found in the timing of p53 overexpression between the intestinal and diffuse type tumours; p53 expression was found in the early stage of intestinal type tumour but only in the late stage of diffuse type tumours (*Table 2*).

Prognostic relevance

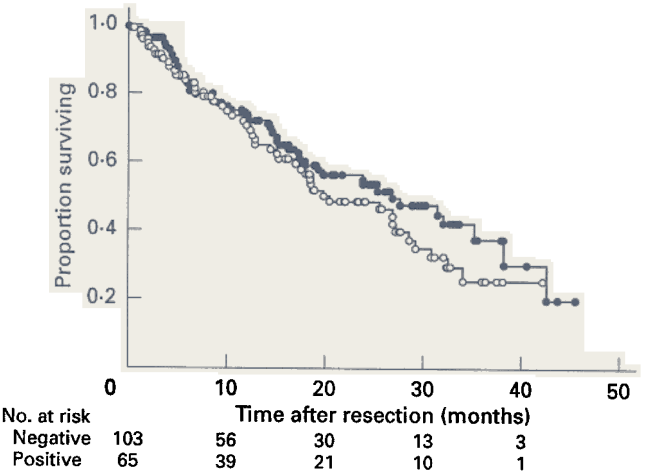
Fig. 2a shows the survival curves of both groups after gastric resection. The survival rate of patients who had p53-positive tumours was lower than that of patients with p53-negative tumours but the difference was not statistically significant ($P = 0{\cdot}400$). The survival of 83 patients with diffuse type adenocarcinomas was analysed separately; a significant difference in survival was seen between patients with p53-positive and p53-negative tumours ($P < 0{\cdot}05$) (*Fig. 2b*). However, the presence of p53 was not a significant prognostic indicator in 72 patients with intestinal type cancer (*Fig. 2c*).

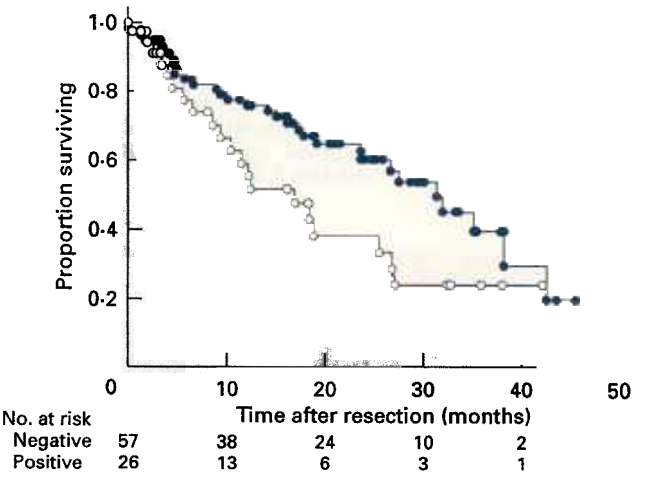
Multivariate analysis of all 168 patients revealed that age, invasion depth and nodal involvement but not p53 expression were independent prognostic variables. Multivariate analysis in 83 patients with diffuse type cancer revealed that only invasion depth was an independent and significant variable.

Discussion

Immunohistochemical techniques have revealed the presence of p53 protein in between 13·3 and 67 per cent of gastric carcinomas[21–32]. The rate of immunostaining for p53 in this study (20·2 per cent) was consistent with the rates of positivity mentioned above. The wide variation in the reported incidence of p53 immunostaining may be due to the various antibodies used in different reports and the difficulty of assessing specimens with low p53 expression. The incidence of p53 abnormalities appears to differ based on the histological type of cancer in previous studies. The present study also showed a higher percentage of p53 expression for intestinal type than diffuse type lesions, the difference was not significant. However, a significant difference in the timing of p53 overexpression between Laurén histological type was observed in this study as well as others[31,32]. This result implicates different carcinogenesis processes between these two types of gastric cancer.

In addition to epidemiological and pathological studies, recent reports have shown that intestinal type and diffuse type gastric cancer are two disease entities with different carcinogenic molecular events[33,34]. Intestinal type cancer, similar to colonic cancer, arises from the progression of





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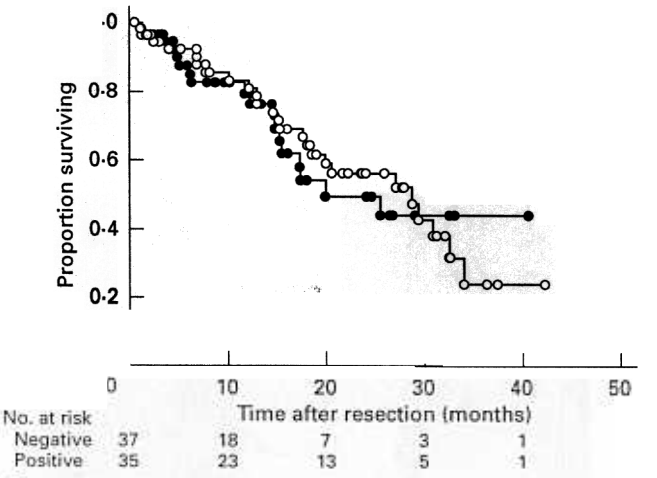

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Fig. 2 Survival of patients after resection of gastric cancer. Cumulative survival curves were constructed for patients with p53-positive (○) or -negative (●) gastric carcinomas by the Kaplan–Meier method. a All 168 patients $P = 0{\cdot}400$ (generalized Wilcoxon test); $P = 0{\cdot}039$ (generalized savage test). b Eighty-three patients with diffuse type cancers. $P = 0{\cdot}045$ (generalized Wilcoxon test); $P = 0{\cdot}039$ (generalized savage test). c Seventy-two patients with intestinal type cancer. $P = 0{\cdot}66$ (generalized Wilcoxon test); $P = 0{\cdot}89$ (generalized savage test)

chronic atrophic gastritis, intestinal metaplasia and severe dysplasia. p53 mutations can be demonstrated in pre-malignant stages and early cancer of intestinal type cancer implying that alterations in p53 are not confined to the late stage for this subtype[25,30]. In diffuse type cancer, regarded as a kind of *de novo* cancer, p53 oncoprotein occurs in the late stage favouring cancer progression and metastases. Kakeji *et al.*[24] have reported that gastric cancer with p53 overexpression has a high potential for metastasizing to lymph nodes[24]. In the present study, there was no significant association between overexpression of p53 and lymph node metastases. However, that most metastatic lesions show positive p53 staining, even in patients with negative staining in primary tumours, corroborates with the finding of Kakaji *et al.*[24]. This could be explained by either a sampling error that might miss the mutation at the primary tumour site or by post-metastatic clinical divergence.

As for prognostic and p53 stainability, many reports have shown that p53 expression can serve as an independent prognostic indicator in the case of breast cancer[13–16]. Evidence for the prognostic relevance of p53 overexpression and gastric cancer is conflicting. Some authors have reported that their p53-positive cases behave more aggressively and have a worse prognosis[22,23,29], but conflicting data exist[26,27]. However, the prognostic relevance has not been compared between different histological types in previous reports and rarely by multivariate analysis. The present report shows that p53 overexpression is associated with significantly poorer survival in patients with diffuse type gastric cancer, suggesting that p53 expression identifies diffuse type carcinomas with a more malignant phenotype. Because multivariate analysis does not reveal p53 expression as an independent prognostic factor, the poorer prognosis for those with tumour expressing p53 may be attributed to a more advanced stage. In contrast, intestinal type gastric cancers are more frequently associated with p53 overexpression but without prognostic relevance. These carcinomas may use another mechanism for tumour progression, such as additional activated oncogenes or deletion of tumour suppressor genes. However, most of the patients present had advanced gastric cancer; p53 expression may still play a significant prognostic role in early gastric cancer when a large number of early cancers are analysed[35,36].

Identification of patients who have resectable high-risk disease is being increasingly taken into account by surgeons and oncologists. A large number of tumour biology-related factors have been investigated, but most have been evaluated retrospectively in paraffin-embedded material. It has been stressed that each factor that is supposed to have an impact on prognosis should be tested prospectively following curative resection in a multivariate analysis. This study evaluated the impact of a single biological factor on overall survival of gastric cancer. Further studies of other potential biological prognostic factors for gastric cancer following curative resection are indicated.

Because intestinal type and diffuse type gastric cancers may be regarded as two disease entities with different carcinogenic processes, it is possible that various biological indicators of tumour aggressiveness may play a different role in predicting the outcome of patients with these two types of gastric cancer[37,38]. It has been found that the ability of c-erbB-2 expression to predict survival is different between well differentiated and poorly differentiated gastric cancers[35]. The present study reveals that p53 expression is a prognostic indication of survival after complete resection of diffuse type gastric cancer but not in intestinal type cancer. In investigating the prognostic relevance of a new biological indicator it is mandatory to analyse these two types of gastric cancer separately.

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