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Prognostic significance of p53 expression in relation to DNA ploidy in colorectal adenocarcinoma

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Abstract. p53 expression, DNA ploidy and S-phase fraction were analysed retrospectively in colorectal adenocarcinomas from 293 patients in whom the long-term outcome was known. The frequency of nuclear p53 staining was increased in non-diploid tumours (42%) when compared with diploid tumours (33%). Cytoplasmic p53 positive tumours were more common in the proximal colon (32%) than in the distal sites (21%). In univariate survival analysis, nuclear p53 and cytoplasmic staining were significantly associated with poor prognosis in patients with Dukes' A–C tumours. The patients showing both nuclear and cytoplasmic p53 staining had the poorest survival and the patients with tumours negative in both the nucleus and cytoplasm showed the best prognosis. The patients with tumours positive in the nucleus alone or in the cytoplasm alone presented an intermediate survival. In multivariate survival analyses, nuclear p53 expression, cytoplasmic p53 expression and DNA ploidy were prognostic indicators independent of Dukes' stage and each other. Further analysis suggested that the prognostic importance of cytoplasmic p53 expression was greater in diploid than in non-diploid tumours. We conclude that nuclear p53 expression, cytoplasmic p53 expression and DNA ploidy provide important prognostic information in colorectal adenocarcinomas.

Key words: p53 protein – DNA ploidy – Colorectal cancer – Immunohistochemistry – Flow cytometry

Introduction

Genetic alterations have an important role in the development of malignancy. In colorectal cancer, they are of two general types; specific genetic events and gross alteration in DNA content. Of the genetic events, p53 gene alteration is very common (Vogelstein 1991). Overexpression of p53 demonstrated by immunohistochemistry and flow cytometry occurs in colorectal tumours (Campo et al. 1991; Scott et al. 1991; Remvikos et al. 1990, 1992; Starzynska et al. 1992; Yamaguchi et al. 1992; Bell et al. 1993). Wild-type p53 protein, as a tumour-suppressor, regulates cellular growth and differentiation (Cossman and Schlegel 1991; Vogelstein 1991). Inactivated p53, through deletion, mutation or complexing to other proteins, confers a growth advantage and results in tumour progression (Lane and Benchimol 1990; Cossman and Schlegel 1991; Vogelstein 1991). Due to genetic instability in tumours, it is possible that inactivation (or activation) of a gene locus increases the likelihood of subsequent multiple genetic alterations, non-disjunctions or other mitotic errors (Nowell 1976; Delattre et al. 1989). Analyses of DNA amount and S-phase fraction (SPF) by flow cytometry reflect the total chromosomal content and growth rate of tumour cells. Our previous study on colorectal cancers has shown that cytoplasmic p53 expression is a powerful prognostic factor and is associated with Dukes' stage but not with grade of differentiation (Sun et al. 1992). In the same large series of colorectal adenocarcinomas, we have now investigated the associations of nuclear p53 expression and cytoplasmic p53 expression with some other clinicopathological variables including ploidy and SPF, and, with extended follow-up time, the prognostic significance of p53 expression in relation to these variables.

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Materials and methods

Specimens were obtained from 293 patients with primary colorectal adenocarcinomas diagnosed in the Department of Pathology, University Hospital of Linköping during the years from 1972 to 1986 (Sun et al. 1992). None of the patients had received pre-operative radiotherapy or chemotherapy for the colorectal carcinoma. There were 156 males and 137 females. The mean age was 69 years (range, 33–93 years). There were 105 poximal tumours (1 in the appendix, 83 in the ascending colon and 21 in the transverse colon) and

180 were distal (12 in the descending, 53 in the sigmoid colon and 115 in the rectum). The location was unknown in 8 cases. Forty-three were Dukes' A, 101 were Dukes' B, 94 were Dukes' C, and 50 were Dukes' D. Dukes' stage was unknown in 5 cases. Tumours with expansive and infiltrative borders were 107 and 150, respectively. The growth pattern was unknown in 36 cases. The patients were followed until the end of December 1991, and 134 patients (85 with Dukes' A–C tumours) died from colorectal cancer. Compared with the follow-up data until the end of December, 1990 (Sun et al. 1992), 10 more patients had died within 3 years of operation. Of these, 5 patients had tumours which were nuclear p53 positive; 3 both nuclear and cytoplasm positive, and 2 cytoplasm negative.

In 19 cases, we also examined the presence of p53 in normal tissue taken from the distant resection margin, which was histologically free from tumour. None of the normal specimens had detectable p53 (Sun et al. 1992).

Nineteen fresh tumours were used to determine the effects of fixation and embedding on tissue p53 immunoreactivity with CM1. Identical findings were observed in 13 cases of negative expression, 3 cases with cytoplasmic staining alone, and 1 case with staining in both the nucleus and cytoplasm. One case showed strong nuclear staining alone in the frozen tissue and only weak staining in the fixed specimen. One showed negative reaction in the frozen tissue but weak cytoplasmic staining alone in the fixed specimen (Sun et al. 1992).

Immunohistochemical staining was performed on sections from 293 paraffin-embedded blocks (Sun et al. 1992). Briefly, 5 μm sections were deparaffinized in xylene followed by rehydration in a series of ethanols at 99.5% (twice), 95%, and 80%. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 20 min. Following a short rinse in phosphate-buffered saline (PBS, pH 7.4), the sections were pre-incubated with 10% normal swine serum to block non-specific immunostaining. NCL-p53-CM1 rabbit polyclonal antibody (Novocastra Laboratories, Newcastle, UK), which detects both wild and mutant forms of p53, was applied in 1:100 dilution as recommended by the supplier for 30 min at room temperature after removing the blocking solution. Subsequently, the sections were incubated with swine anti-rabbit immunoglobulins (Dakopatts, Glostrup, Denmark) and rabbit peroxidase and anti-peroxidase (PAP) (Dakopatts), 30 min for each step. The slides were washed in PBS between each incubation. The peroxidase reaction was performed for 8 min, using 0.05% 3,3-diaminobenzidine tetrahydrochloride solution (Sigma Chemical, St. Louis, Mo., USA) in PBS containing 0.02% hydrogen peroxide. The sections were counterstained with light haematoxylin for 1 min, dehydrated in a series of ethanols, cleared in xylene and mounted under a coverslip. Sections known to stain positively were included in each run, receiving either primary antibody or PBS, as positive and negative controls. We registered nuclear p53 positivity when the nucleus was stained regardless of cytoplasmic expression, and cytoplasmic positivity when the cytoplasm was stained regardless of nuclear expression, irrespective of the percentage of positive cells. The slides were examined and scored independently by two of us (X-F S and HZ) without information of the pathological and clinical data.

In the previous study (Sun et al. 1992), we have compared CM1 staining on frozen tissue and the corresponding fixed, paraffin-embedded samples, identical findings were observed in 17 cases. One case showed strong nuclear staining only in the frozen tissue and only weak staining in the fixed specimen. One showed negative reaction in the frozen tissue but weak cytoplasmic staining only in the fixed specimen.

Sections 50 μm thick from the same paraffin-embedded tissue used for the PAP method were deparaffinized with xylene, then rehydrated stepwise in 99.5, 95, 70 and 40% ethanol. Finally they were washed twice in distilled water. The samples were treated with 0.4% trypsin (Sigma) in a citrate buffer for 24 h in a 37° C shaking waterbath. After filtration through a nylon mesh, the suspension was stained with propidium iodide and kept from light in an ice bath.

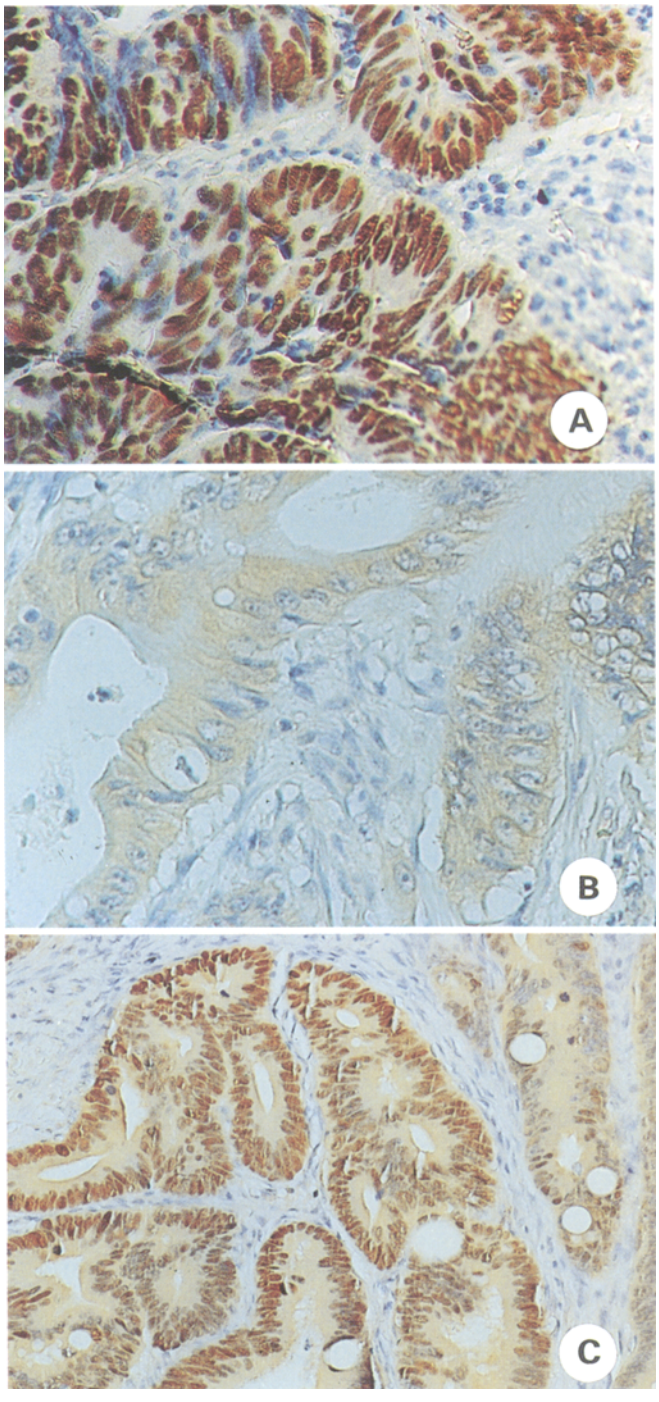

Fig. 1 A–C. Immunostaining of colorectal adenocarcinomas using NCL-p53-CM1 anti-p53 oncoprotein antibody. The sections received a light haematoxylin counterstain. **A** Positive nuclear p53 expression without cytoplasmic staining, ×200, **B** positive cytoplasmic p53 expression without nuclear staining, ×200, **C** positive nuclear and cytoplasmic p53 expression, ×200

The cell suspensions were analysed with a FACScan flow cytometer (Becton-Dickinson, USA) Histrograms including 15000 cells, and DNA peaks with a mean coefficient of variation of 6.5% were recorded. Normal diploid cells from the same specimens were used as internal controls. Tumours were defined as diploid if a single $G_{0/1}$ peak was obtained, and as non-diploid if more than one distinct $G_{0/1}$ peak occurred. SPF was estimated by using a

rectangular model. The number of S-phase cells was calculated by multiplying the number of channels between the $G_{0/1}$ and G_2/M peaks by the mean number of cells per channel in a part of the S-phase interval judged as representative by the operator. Peaks overlapping a part of the S-phase interval could be excluded when the SPF was calculated. A simple method for background correction was used by subtraction of a constant estimated in each of the histograms. The mean number of registrations per channel in an area to the right of the G_2/M peak was calculated and subtracted from the mean number of registrations per channel in the S-phase interval. In most of the statistical analyses, the SPFs were divided into three categories; low (<5%), moderate (5–10%) and high (>10%), respectively.

Chi-square tests for 2×2 tables and for contingency tables with ordered categories were used to test the relationships between p53 expression and clinico-pathological variables (Armitage and Berry 1987). Cox's proportional hazards model was used to estimate and test the influence of p53 expression, DNA ploidy, SPF and other clinico-pathological variables on prognosis (Cox 1972). In multivariate analyses, we also tested for interactions between the various main factors; only significant interactions were reported. The curves describing survival were computed according to the method of Kaplan and Meier (Kaplan and Meier 1958). In the survival analyses, only deaths due to colorectal cancer were considered as uncensored observations. All p-values cited were two-sided, and p-values less than 5% were judged as statistically significant.

Results

Among the 293 tumours, 114 (39%) showed p53 positive expression in the nucleus (Fig. 1A) and 73 (25%) in the cytoplasm (Fig. 1B). Of these, 43 (15%) showed both nuclear and cytoplasmic staining (Fig. 1C). The remaining 149 (51%) cases were negative in both the nucleus and cytoplasm.

A satisfactory DNA histogram was obtained from 279 cases. One hundred and thirty-five (48%) tumours were diploid, and 144 (52%) were non-diploid. Excluding 49 cases in which SPF could not be estimated, the mean SPF was 9.2% (SD = 5.6%). Sixty-four (28%) tumours had a SPF or less than 5%, 79 (34%) had a SPF of between 5% and 10%, and 87 (38%) had a SPF of greater than 10%.

The associations of p53 expression with clinico-pathological variables are summarized in Table 1. The tumours with cytoplasmic p53 staining were more common in the proximal colon than in distal sites ($p = 0.035$), while no correlation of nuclear p53 expression with tumour site was found ($p = 0.82$). The frequency of nuclear p53 staining tended to be increased in non-diploid tumours when compared with diploid ones, although the difference did not reach statistical significance ($p = 0.12$). We did not find any significant relationships between nuclear p53 expression and SPF ($p = 0.40$), between cytoplasmic p53 expression and DNA ploidy ($p = 0.97$) or SPF ($p = 0.47$). No significant relationships between p53 expression and patients' sex, age or tumour growth pattern were obtained, although cytoplasmic p53 staining tended to be more common in older patients ($p = 0.11$).

In univariate survival analyses, nuclear p53 staining ($p = 0.027$, Fig. 2) and cytoplasmic p53 staining ($p = 0.0005$, Fig. 3) were significantly associated with poor prognosis in the 238 patients with Dukes' A–C tumours. It is noted from Figs. 2 and 3 that the prognostic effect of cytoplasmic staining was evident early during the follow-up while the prognostic value of nuclear staining appeared clearly 3 years after operation. As Fig. 4 shows, the patients with Dukes' A–C tumours showing both nuclear and cytoplasmic staining had the poorest survival, and the patients with tumours negative in both the nucleus and cytoplasm showed the best prognosis

Table 1. p53 expression in relation to sex, age, site, growth pattern, DNA ploidy and S-phase fraction (SPF) in colorectal adenocarcinomas

Variable Category	Nuclear expression				Cytoplasmic expression		
	No.	Negative	Positive	P	Negative	Positive	P
Sex				0.68			0.56
Male	156	62%	38%		74%	26%	
Female	137	60%	40%		77%	23%	
Age				0.25*			0.11*
≤59	52	67%	33%		85%	15%	
60–69	92	63%	37%		76%	24%	
70–79	93	57%	43%		70%	30%	
≥80	56	59%	41%		73%	27%	
Site				0.82			0.035
Proximal	105	62%	38%		68%	32%	
Distal	180	61%	39%		79%	21%	
Growth pattern				0.47			0.93
Expansive	107	66%	34%		74%	26%	
Infiltrative	150	62%	38%		73%	27%	
DNA ploidy				0.12			0.97
Diploid	135	67%	33%		75%	25%	
Non-diploid	144	58%	42%		75%	25%	
SPF (%)				0.40*			0.47*
<5	64	64%	36%		77%	23%	
5–10	79	72%	28%		72%	28%	
>10	87	59%	41%		77%	23%	

* Test for trend

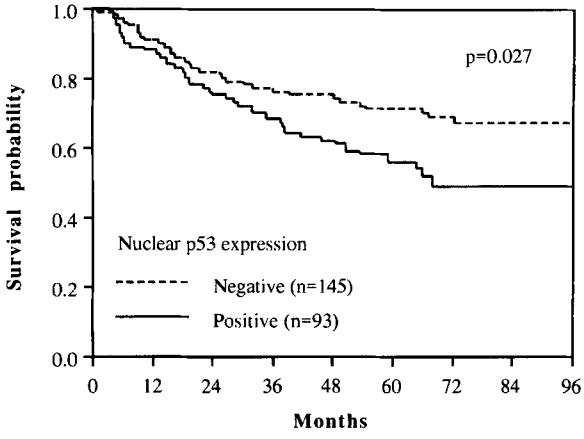

Fig. 2. Nuclear p53 expression in relation to prognosis in patients with Dukes' stage A–C colorectal adenocarcinomas

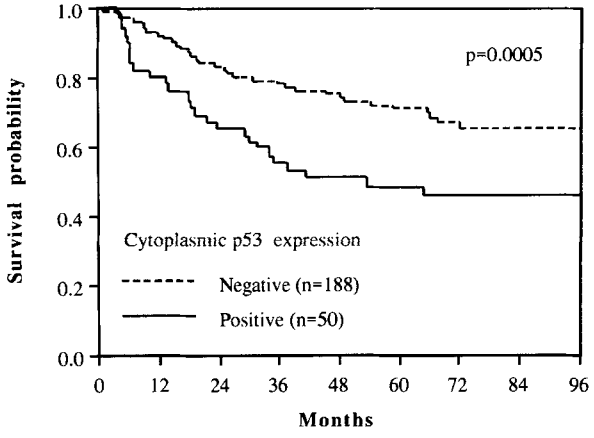

Fig. 3. Cytoplasmic p53 expression in relation to prognosis in patients with Dukes' stage A–C colorectal adenocarcinomas

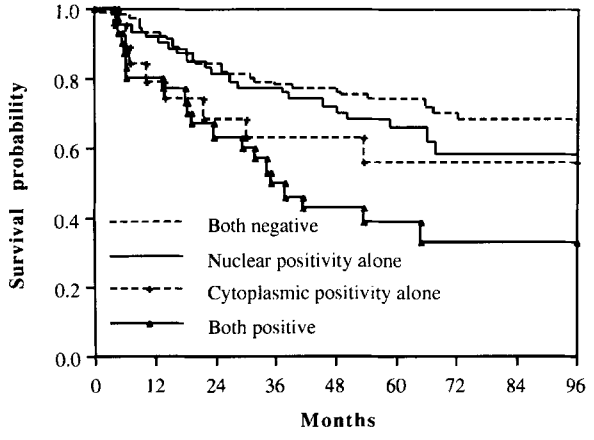

Fig. 4. p53 negative expression in both the nucleus and cytoplasm ($n=126$), nuclear positivity without cytoplasmic staining ($n=62$), cytoplasmic positivity without nuclear staining ($n=19$) and positivity in both the nucleus and cytoplasm ($n=31$) in relation to prognosis in patients with Dukes' stage A–C colorectal adenocarcinomas

Table 2. Multivariate analysis of nuclear p53 expression, cytoplasmic p53 expression, DNA ploidy and Dukes' stage in relation to prognosis in colorectal adenocarcinomas

Variable Category	Cancer death No.	rate ratio	95% CI	*P*
Nuclear p53 expression				0.044
Negative	141	1.0	–	
Positive	87	1.6	1.0–2.5	
Cytoplasmic p53 expression				0.0076
Negative	180	1.0	–	
Positive	48	2.0	1.2–3.2	
DNA ploidy				0.012
Diploid	113	1.0	–	
Non-diploid	115	1.8	1.1–2.9	
Dukes' stage				<0.0001*
A	41	1.0	–	
B	98	2.8	1.0–8.1	
C	89	7.4	2.6–20.7	

* Test for trend

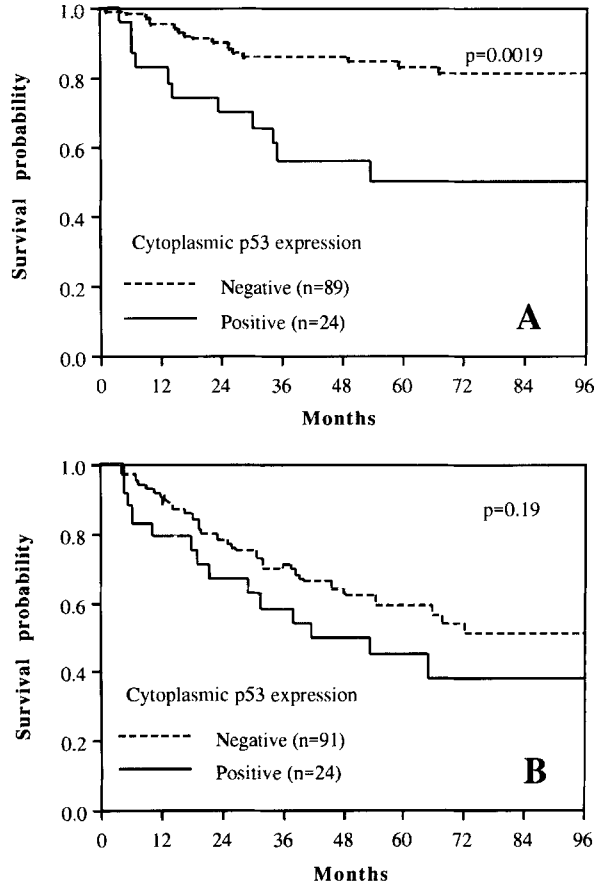

Fig. 5A, B. Cytoplasmic p53 expression in relation to prognosis in patients with diploid (**A**) and non-diploid (**B**) colorectal adenocarcinomas

($p=0.0002$). The patients with tumours positive in the nucleus alone or in the cytoplasm alone presented an intermediate survival.

Multivariate analysis showed that nuclear p53 expression ($p=0.044$), cytoplasmic p53 expression ($p=0.0076$),

DNA ploidy ($p=0.012$) and Dukes' stage ($p<0.0001$) were independent prognostic factors (Table 2). Tumour SPF, grade of differentiation, growth pattern, site, age and sex did not contribute significant prognostic information.

Further multivariate analyses, however, suggested that correlations with prognostic significance of cytoplasmic p53 expression differed significantly between the patients with diploid tumours and the patients with non-diploid tumours ($p=0.020$). We therefore did separate analyses of the relationship between cytoplasmic p53 expression and survival in the DNA subgroups of tumours. These analyses showed that cytoplasmic p53 expression was significantly associated with prognosis in patients with diploid tumours ($p=0.0019$, Fig. 5A) but not in those with non-diploid tumours ($p=0.19$, Fig. 5B). There was no significant difference between diploid and non-diploid tumours concerning the association of nuclear p53 expression with survival ($p=0.75$).

Discussion

Our results showed that nuclear p53 staining tended to be more common in non-diploid than in diploid tumours, which is in line with the findings of Remvikos et al. (1990). But, like Campo et al. (1991), we did not find that nuclear p53 expression was related to SPF. This suggested that other mechanisms, besides the p53 gene, were likely to be implicated in the uncontrolled cancer growth.

Previous analyses have suggested that nuclear p53 staining is more common in the distal colon and rectum (Scott et al. 1991; Starzynska et al. 1992; Bell et al. 1993). In the present study, we did not find a significant association between nuclear p53 expression and tumour site, but cytoplasmic p53 positive expression was significantly more frequent in proximal tumours. It has been demonstrated that there are differences in the epidemiological behaviours between proximal and distal large bowel tumours (Weisburger 1991). Cytoplasmic p53 protein may be involved in different biological mechanisms in the initiation and progression of proximal and distal colorectal carcinomas.

In our previous analyses, we found that cytoplasmic p53 staining predicts an unfavorable outcome, but nuclear p53 positivity is not significantly related to patient survival (Sun et al. 1992). As we have extended the follow-up time in the present study, survival analysis revealed that nuclear p53 expression came to be a significant prognostic indicator. After 3 years following operation the mortality of patients with nuclear p53 positive tumours was increased compared with that of the patients with other types of immunostaining. In two other studies with a mean follow-up time less than 3 years, nuclear p53 protein did not show a prognostic significance in colorectal cancers (Scott et al. 1991; Bell et al. 1993), whereas Kern et al. (1989) found that 17p deletion was associated with lower survival rate when the mean follow-up was 40.5 months. We are continuing to follow-up the patients to clarify the prognostic significance of p53 in different follow-up periods.

In the multivariate analyses, nuclear p53 expression, cytoplasmic p53 expression and DNA ploidy were prognostic indicators independent of Dukes' stage and each other. Analyses of the prognostic significance of p53 expression in DNA subgroups of tumours showed that the relationship between cytoplasmic p53 expression and survival was significantly stronger in diploid than in non-diploid tumours. A possible hypothesis is that more genetic abnormalities occur in non-diploid tumours and these may be involved in patient survival (Delattre et al. 1989; Sun et al. 1991; Bell et al. 1993), these alterations might interact with p53 and influence the prognostic significance of cytoplasmic p53.

To summarize, the first main finding in the present study is that the mortality of patients with nuclear p53 positive tumours tended to increase more rapidly 3 years after operation; as the follow-up time extended, nuclear p53 protein became a valuable prognostic factor. Secondly, the nuclear and cytoplasmic p53 oncoproteins seemed to be involved in differing tumour behaviours, such as tumour location, DNA ploidy and prognosis in DNA subgroups of tumours. Our previous analyses revealed that nuclear and cytoplasmic p53 proteins show different immunostaining reactivity with different anti-p53 antibodies CM1 and PAb 1801, and that cytoplasmic p53 positivity but not nuclear positivity is related to Dukes' stage (Sun et al. 1992). The reason for these differences are not clear. However, some studies have suggested that different mutant p53 proteins have different properties with respect to their half life, the ability to bind heat shock proteins, to transform culture cells in combination with an activated *ras* gene, to inactivate wild-type p53 protein, or to abrogate control of gene transcription (Harris and Hollstein 1991). The third principle finding of our study is that nuclear p53 expression, cytoplasmic p53 expression and DNA ploidy are independent prognostic factors. Thus, the combination of immunohistochemistry and flow cytometry may provide an important insight into the biological behaviour of colorectal adenocarcinoma.

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