Evaluation of p53 protein expression as a marker for long-term prognosis in colorectal carcinoma

J-WR Mulder[1], IO Baas[1], MM Polak[1], SN Goodman[2] and GJA Offerhaus[1]

[1]Department of Pathology, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam Zuid-Oost, The Netherlands; [2]Oncology Center, Division of Biostatistics, The Johns Hopkins Medical Institutions, 550 N Broadway, Baltimore MD 21205, USA.

Summary Mutation of the p53 gene is reported to be of prognostic importance in colorectal carcinomas. Immunohistochemical staining of the accumulated p53 gene product may be a simple alternative for p53 mutation analysis. Previous studies addressing the prognostic importance of p53 expression, however, yielded contradictory results. Therefore, we evaluated the importance of p53 expression as a marker for long-term prognosis in a well-characterised study population of 109 colorectal carcinomas. After antigen retrieval with target unmasking fluid (TUF), immunostaining of p53 was performed with both monoclonal antibody DO7 and polyclonal antibody CM1. Objective quantification of the p53 signal was assessed by a computerised image analyser. p53 expression was higher in non-mucinous tumours than in mucinous tumours (p53 labelling index = 30% and 17% respectively, $P = 0.05$), and in metastatic tumours compared with non-metastatic tumours (p53 labelling index = 37% and 22% respectively, $P = 0.05$). Other histopathological features were not related to p53 expression. In multivariate analysis, Dukes' stage ($P = 0.02$) and histological grade ($P = 0.05$) stood out as independent markers for prognosis. p53 expression was not an independent marker for prognosis. At present, p53 expression is not a useful marker for long-term prognosis. Further insight into the relationship between p53 mutations and p53 expression is needed to elucidate more precisely the clinical relevance of p53 alterations.

Keywords: p53; long-term; prognosis; colorectal; carcinoma

The p53-suppressor gene is the most frequently altered gene in solid human malignancies (reviewed in Lane, 1992; Levine, 1992a, 1992b; Oren, 1992; Vogelstein and Kinzler, 1992). It is located on the short arm of chromosome 17 in the region 17p13 and encodes a 53 kDa nuclear phosphoprotein that serves as a transcription factor (Kern et al., 1992; El-Deiry et al., 1993). The p53 protein indirectly regulates cell growth and inhibits cells with mutagenic damage from entering the S-phase by arresting the cell cycle in G_1, during which DNA repair can proceed.

In colorectal cancer, p53 mutations are frequently accompanied by allelic loss of 17p (Baker et al., 1989; Rodrigues et al., 1990). Both p53 mutations and allelic deletion of 17p occur late in tumour progression (Baker et al., 1990) and are reported to have prognostic value after surgery (Kern et al., 1989; Laurent-Puig et al., 1992; Offerhaus et al., 1992; Hamelin et al., 1994). However, both detection of p53 mutations at the DNA level and detection of allelic deletion of 17p by restriction fragment length polymorphism (RFLP) analysis are cumbersome procedures and therefore not feasible in routine diagnosis.

Recent reports describe a strong relation between p53 gene mutations and mutant p53 protein expression (Rodrigues et al., 1990; de Angelis et al., 1993; Baas et al., 1994). The mutant p53 protein is characterised by a conformational change resulting in prolonged half-life and stability, enabling its detection by routine immunohistochemical (IHC) techniques (Finlay et al., 1988). Therefore, immunostaining of the p53 protein may be an important surrogate test for p53 mutation analysis.

In solid neoplasms including carcinomas of the breast (Barnes et al., 1993), stomach (Martin et al., 1992; Starzynska et al., 1992), lung (Quinlan et al., 1992), ovary (Bosari et al., 1993) and pancreas (DiGuiseppe et al., 1994), p53 expression has been correlated with shortened survival. In colorectal carcinomas, however, a correlation between survival and nuclear p53 expression has not been consistently observed (Scott et al., 1991; Remvikos et al., 1992; Starzynska et al.,

1992; Sun et al., 1992; Yamaguchi et al., 1992; Bell et al., 1993; Bosari et al., 1994; Nathanson et al., 1994). The contradictory results of these studies might be partly due to the variability in IHC techniques used. Moreover, most follow-up studies lacked statistical power owing to relatively small patient populations or limited follow-up periods.

Therefore, in this study we analysed the value of p53 protein expression for long-term prognosis in a large, well-characterised study population with over 20 years of follow-up. p53 expression was evaluated by two different anti-p53 antibodies, which in a previous study stood out as being most accurate for p53 protein detection and association with p53 gene mutation (Baas et al., 1994). p53 expression was objectively scored by a computerised image analyser. In addition, we evaluated the relationship between p53 expression and other histopathological parameters known to be of importance in colorectal cancer.

Materials and methods

Study population and follow-up

The original study population consisted of 155 patients with colorectal carcinoma, operated on between 1967 and 1974 in the University Hospital of Leiden. The study population had previously undergone extensive research for a large number of histopathological parameters which have a bearing on tumour biology and prognosis (Bloem, 1983; Offerhaus et al., 1991). For the present p53 immunostudy, tissue blocks were available from 109 patients only. These patients did not differ significantly from the original 155 with respect to age, sex or the histopathological parameters. Histopathological parameters were determined by review of slides; location of the tumour and macroscopic aspect, together with the patient characteristics, were collected from the medical records by review of charts.

In the p53-tested cohort of 109 patients, there were 56 men and 53 women; the median age was 66 years (mean age 65 years, s.d. = 10 years, range 25-96 years). Twenty-two tumours were located in the caecum or ascending colon, eight in the transverse colon or splenic flexure, 46 in the descending colon or sigmoid and 33 tumours in the rectum. Tumours

were staged according to the modified Dukes' classification (Dukes, 1932; Turnbull et al., 1967). Eighteen patients had a Dukes' A carcinoma (confined within the muscularis propria), 61 patients had a Dukes' B carcinoma (extension through the muscularis propria into the pericolic fat), 27 patients had a Dukes' C carcinoma (positive regional lymph nodes without distant metastases), and three patients had a Dukes' D carcinoma (either invasion of adjacent organs or evidence of distant metastases). Twenty carcinomas were well differentiated, 67 were moderately differentiated and 22 were poorly differentiated. Twenty-nine of the 109 tumours were mucinous carcinomas (defined as at least 30% of the volume being occupied by mucine lakes) (Mecklin et al., 1986). Of those cases in which the macroscopic aspect was reliably reported, 34 tumours showed exophytic growth and 55 tumours showed ulcerative growth. Fourteen tumours showed 'Crohn's-like' lymphocytic infiltration (Jass, 1986; Graham and Appelman, 1990); in five cases the presence or absence of lymphocytic infiltration was not evaluable. In 102 tumours vasoinvasion of tumour cells was studied by Van Gieson's elastic stain and a factor VIII immunoperoxidase method for the localisation of endothelial cells (Mukai et al., 1980; Muller et al., 1989; Offerhaus et al., 1991).

Follow-up was obtained through physician contact and ended on 30 September 1993.

Immunohistochemistry for p53

On 109 paraffin-embedded specimens, routine immunostaining was performed as reported previously (Baas et al., 1994), using target unmasking fluid ('TUF'; Kreatech Technology, Amsterdam, The Netherlands) to enhance antigen retrieval (van den Berg et al., 1993). Both rabbit polyclonal antibody CM1 (Novacastra laboratories, Newcastle upon Tyne, UK) and mouse monoclonal antibody DO7 (Dakopatts, Glostrup, Denmark) against the p53 protein served as primary antibodies (Baas et al., 1994). Further staining was with the streptavidin–horseradish peroxidase (HRP)–ABC method (Vectastain, Vector Laboratories, Burlingame, CA, USA), and the chromagen was diaminobenzidine (DAB). Nuclear counterstaining was performed with methyl green, enabling p53 protein quantification by an image analyser (Baas et al., 1994). Staining was controlled by omission of the primary antibody.

Image analysis

The CAS 200 image analysis system consists of a conventional microscope with mounted television camera which is linked to a computer and colour monitor. The ER PR software program enabled measurement of the total area of positive nulcear staining in any selected microscopic field, while the methyl green nuclear counterstain enabled measurement of the total nuclear area. The ratio expressed as p53 labelling index (LI) gave an objective value for the percentage of positive-staining nuclei. Baseline was set on p53-negative normal mucosa. Negative stromal elements were controlled for by computing the mean p53 LI for each slide in at least five representative fields at 400 × magnification, containing between 100 and 250 tumour nuclei (Baas et al., 1994).

Statistical analysis

Statistical analysis was performed with JMP software (SAS Institute, Cary, NC, USA). For survival analysis p53 expression was divided into three groups: (1) no nuclear p53 expression (LI <1%), (2) low nuclear p53 expression (LI 1–30%), and (3) high nuclear p53 expression (LI > 30%). This tripartition is based on the results of previous studies by our group (Baas et al., 1994). Survival analysis for the other histopathological parameters was assessed in both the p53-tested cohort and the original cohort. One patient died within 30 days of surgery, and was therefore excluded from survival analysis. Kaplan–Meier survival curves were calculated and

tested for significance by an univariate log-rank statistic. These curves included only colorectal cancer-related deaths as events. Deaths from other causes were treated as censored events at time of occurrence. One patient was lost to follow-up after 11.8 years and treated as a censored event from that time. The independent prognostic value of parameters was tested using the multivariate Cox regression model. Correlation between p53 expression and histopathological parameters was tested using a t-test statistic or analysis of variance (ANOVA) for multiple means.

Results

p53 expression

p53 immunostaining was initially evaluated by conventional light microscopy by two authors who were blinded for other

Table I p53 expression by immunostaining with MAb DO7 and PAb CM1

Percentage positive cells	Estimation by conventional light microscopy				Quantification by computerised image analysis		
	DO7		CM1		Labelling index (%)	DO7	
	n	%	n	%		n	%
<1	23	21	35	32	LI <1	31	28
1–30	17	16	25	23	LI 1–30	35	32
>30	69	63	49	45	LI >30	43	40
	109	100	109	100		109	100

p53 protein positivity is evaluated by conventional light microscopy for MAb DO7 and PAb CM1. p53 labelling index (LI) is assessed on a computerised image analyser for p53 staining with MAb DO7.

Table II Association between IHC p53 expression with MAb DO7 and different histopathological parameters (mean p53 LI = the average of the labelling indices in the given subgroup)

	n	Mean p53–LI (%)	P
Sex			
Male	56	27	
Female	53	26	>0.2
Age (years)			
<66	54	25	
>66	55	28	>0.2
Location			
Caecum ascending colon	22	21	
Transverse colon splenic flexure	8	28	
Descending colon sigmoid	46	29	
Rectum	33	26	>0.2
Dukes' stage			
A	18	26	
B	61	21	
C D	30	37	0.05
Differentiation grade			
Good	20	23	
Moderate	67	27	
Poor	22	27	>0.2
Mucus content			
Mucinous	29	17	
Non-mucinous	80	30	0.05
Macroscopic aspect			
Exophytic	34	30	
Ulcerative	55	27	>0.2
Lymphocytic infiltration			
Absent	90	27	
Present	14	27	>0.2
Vasoinvasion			
Absent	56	29	
1 vessel	25	18	
>1 vessel	21	34	0.13

variables. p53 positivity was restricted to the nuclei of the cells of malignant glands. Normal colonic mucosa expressed no p53 protein. The results of the p53 immunostaining are listed in Table I. A high correlation was found between the results of the p53 detection with MAb DO7 and pAb CM1 ($P < 0.0005$). Therefore, objective quantification of p53 expression with a CAS 200 image analyser was performed only on the DO7-stained specimens.

Table I shows that the results of conventional evaluation of p53 expression slightly differ from the amount of p53 protein when quantified by a computerised image analyser. Thirty-one (28%) carcinomas showed no p53 expression ($LI < 1\%$), 35 (32%) carcinomas showed low p53 expression ($LI\ 1-30\%$) and 43 (40%) carcinomas showed high p53 expression ($LI > 30\%$).

p53 expression and histopathological parameters

The associations between p53 expression and histopathological parameters are listed in Table II. A weak significant

overall increase in p53 expression with advancing Dukes' stage was observed ($P = 0.05$). Mucinous tumours expressed significantly less p53 protein than non-mucinous tumours ($P = 0.05$). No significant difference in p53 expression with regard to sex, age, macroscopic aspect, lymphocytic infiltration or vasoinvasion by tumour cells was observed. Results did not change significantly when low p53 expression ($LI\ 1-30\%$) was regarded as negative, or when a subdivision into p53-negative ($LI < 1\%$) vs p53-positive ($LI > 1\%$) carcinomas was used.

Survival analysis

The results of the univariate survival analysis in the p53-tested study group and the original complete cohort are listed in Table III. The median period between surgery and death or last physician contact was 7.3 years in both groups. During follow-up, in the complete cohort 57 (37%) patients died of colorectal carcinoma and 65 (42%) patients died of causes unrelated to colorectal cancer. In the p53-tested

Table III Prognostic importance of histopathological parameters tested by univariate log-rank analysis and the multivariate Cox regression model. The risk ratios at each level use as a reference the previous level, not the baseline level (RR = relative risk)

	Complete cohort 10 year			p53-tested cohort 10 year		
	n	surv (%)	P	n	surv (%)	P
Univariate analysis						
Sex						
Male	81	56		55	55	
Female	73	62	>0.2	53	59	>0.2
Age						
<median	77	60		54	58	>0.2
>median	77	58	>0.2	54	56	
Location						
Caecum/ascending colon	31	59		22	58	
Transverse colon/splenic flexure	12	57		8	63	
Descending colon/sigmoid	64	62		45	53	
Rectum	47	54	>0.2	33	58	>0.2
Dukes' stage						
A	28	82		18	73	
B	90	61		61	60	
C/D	36	37	<0.001	29	41	0.03
Differentiation grade						
Good	27	76		20	74	
Moderate	95	60		66	57	
Poor	32	41	0.005	22	40	0.04
Mucus content						
Mucinous	42	64		29	73	
Non-mucinous	112	57	>0.2	79	51	0.2
Macroscopic aspect						
Exophytic	47	73		34	71	
Ulcerative	77	54	0.04	55	51	0.13
Lymphocytic infiltration						
Absent	120	54		90	54	
Present	26	75	0.04	14	63	>0.2
Vasoinvasion						
Absent	74	60		56	64	
1 vessel	34	57		25	67	
>1 vessel	25	35	0.03	21	38	0.08
p53 expression (%)						
LI <1				31	45	
LI 1-30				35	74	
LI >30				43	52	0.08
	n	RR	P	n	RR	P
Multivariate analysis						
Dukes' stage						
A	28	1.0		18	1.0	
B	90	2.3		61	1.5	
C/D	36	2.1	<0.001	29	1.9	0.02
Differentiation grade						
Well	27	1.0		20	1.0	
Moderate	95	1.6		66	1.5	
Poorly	32	1.9	0.01	22	1.9	0.05

cohort these numbers were 42 (39%) and 48 (44%) respectively.

In the complete cohort, increase in Dukes' stage (Figure 1a), poorer grade of differentiation, ulcerative growth, lymphocytic infiltration and vasoinvasion was related to worse prognosis. In the smaller p53 study subset, only Dukes' stage (Figure 1b) and grade of differentiation remained significant. The amount of p53 expression in carcinomas showed a tendency towards an association with patient survival ($P = 0.08$), but the pattern of this relationship is difficult to interpret: the highest and lowest p53 categories showed the poorest survival, whereas the intermediate p53 category showed the best prognosis (Figure 1c).

In the complete cohort, multivariate analysis showed that only Dukes' stage ($P = <0.001$) and differentiation grade ($P = 0.01$) were independent markers for prognosis. These parameters were also independent predictors of prognosis in the smaller p53-tested cohort (Dukes' stage, $P = 0.02$; differentiation grade, $P = 0.05$) (Table III).

Discussion

The prognosis after seemingly curative resection of colorectal carcinoma depends largely on the absence or presence of occult metastases, often accounting for mortality. Prediction of outcome is currently based mainly on the stage of colorectal carcinoma at time of resection. However, patients with tumours of the same stage often show dramatically different outcome. Therefore, more specific prognostic markers would provide a rationale to adjust different therapeutic approaches. Alterations of the p53 tumour-suppressor gene are potentially such a marker (Hamelin et al., 1994), and immunostaining of the p53 protein product could be a valuable test for p53 gene alterations (Rodrigues et al., 1990; de Angelis et al., 1993; Baas et al., 1994).

Previous studies addressing the prognostic value of p53 were mostly restricted to short-term follow-up, and in particular the IHC studies yielded variable results (Table IV). This variability might come from several causes. First of all, these study groups may not always be comparable. Moreover, the use of various antibodies against different epitopes of the p53 protein, and sometimes the use of antigen retrieval systems, may also account for some of the variability in the percentages of p53 positivity seen among the different studies (Table IV) (van den Berg et al., 1993; reviewed in Wynford-Thomas, 1992; Hall and Lane, 1994). We previously evaluated various procedures and six different p53 antibodies in relationship with underlying p53 changes and selected the two most accurate procedures for p53 protein detection for use in this study (Baas et al., 1994). Objective quantification of p53 expression was achieved by use of a computerised image analyser. Optimised IHC techniques combined with computerised quantification yielded p53 positivity in 72% of the carcinomas (Table I), a percentage that exceeds that of all previous studies (Table IV).

In our study, p53 expression was higher in metastatic carcinomas (LI = 37%) than in non-metastatic Dukes' B carcinomas (LI = 21%). However, this phenomenon is not consistently observed in other studies (Campo et al., 1991; Scott et al., 1992; Purdie et al., 1991; Remvikos et al., 1992; Starzynska et al., 1992; Sun et al., 1992; Bell et al., 1993; de Angelis et al., 1993; Bosari et al., 1994; Mulder et al., 1995). As in other studies (Campo et al., 1991; Hanski et al., 1992), mucinous carcinomas exhibited significantly less p53 protein than non-mucinous carcinomas, suggesting more p53 mutations in non-mucinous carcinomas. Together with other molecular aspects (Kern et al., 1989; Laurent-Puig et al., 1991) and similar findings in the ovary (Enomoto et al., 1991), this finding suggests that mucinous tumours may be biologically different. No correlation was found between p53 expression and the other histopathological parameters. In this study, we did not observe a difference in p53 positivity between right- and left-sided carcinomas. This contrasts with some studies in which p53-positive tumours were predomin-

antly found in the distal part of the large bowel (Scott et al., 1991; Remvikos et al., 1992; Starzynska et al., 1992; Bosari et al., 1994), but is concordant with other studies (Purdie et al., 1991; Hanski et al., 1992; Yamaguchi et al., 1992; Bell et al., 1993; Nathanson et al., 1994). The other results fit in the general picture derived from other studies (Campo et al.,
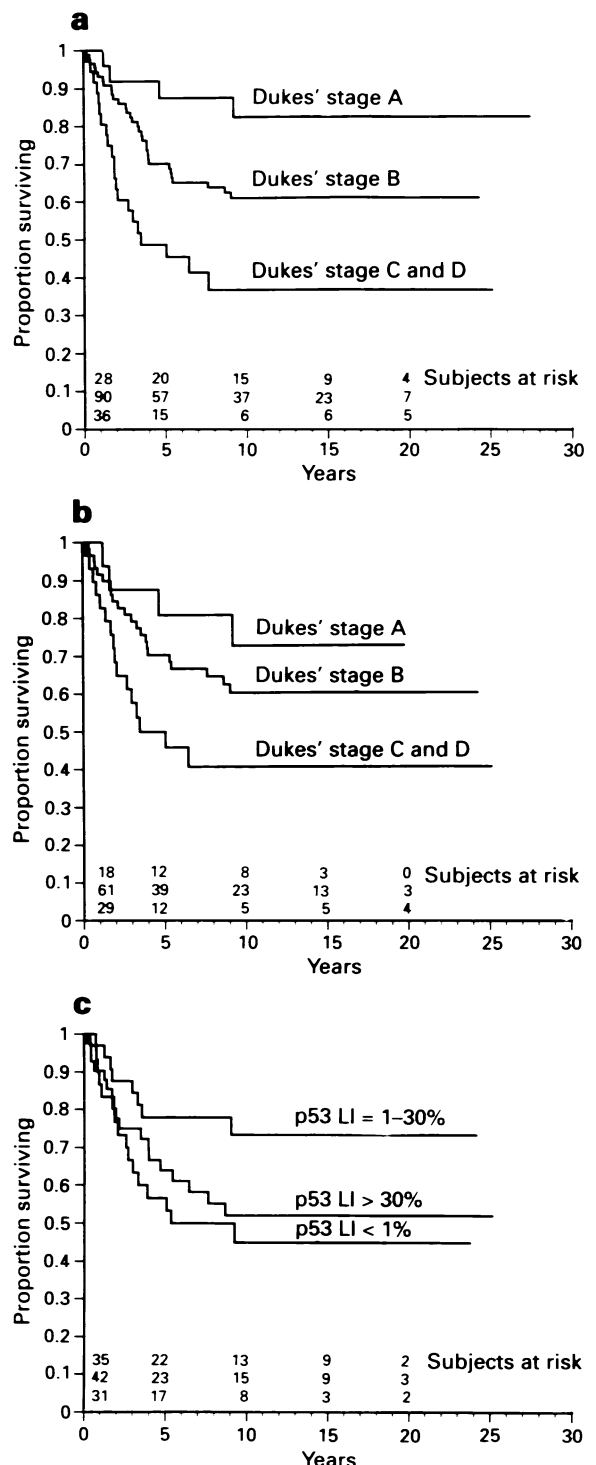

Figure 1 Kaplan-Meier survival curves according to Dukes' classification in the original complete cohort (a) and the smaller p53-tested study group (b), and according to p53 expression in p53-tested group (c). Prognostic importance was tested by univariate log-rank statistic. Subjects at risk are indicated in the figures. Increase in Dukes' stage was significantly related to survival in both the complete cohort and the p53-tested group ($P<0.001$ and $P = 0.03$ respectively). p53 expression showed a tendency towards a relationship with survival ($P = 0.08$), but the pattern of the relationship is difficult to interpret: the highest and lowest p53 categories showed the poorest survival, whereas the intermediate p53 category showed the best prognosis.

Table IV Survey of recent studies addressing p53 expression in colorectal carcinomas (crc) by immunostaining. When more than one antibody was evaluated, the antibody (Ab) yielding the highest staining percentage is listed. When studied, the importance for prognosis is listed

Reference	Per cent of crc expressing p53 (Ab)	Antigen enhancement	Other antibodies evaluated	n	Follow-up in years Mean range	Prognostic value Univariate P	Prognostic value Multivariate P
This study	72 (DO7)	TUF[a]	CM1	109	7.3[b] 0-28	NS	NS
Starzynska et al. (1992)	46 (CM1)	—	—	107	<1 0-1	<0.001	—
Bell et al. (1993)	45 (421)	—	240/1801	100	3 0-8	NS	—
Yamaguchi et al. (1992)	61 (1801)	—	—	100	3 0.5-4	0.01	<0.05[c]
Scott et al. (1991)	42 (421)	—	—	52	3 1-7	NS	—
Remvikos et al. (1992)	60 (240)	—	421/1801	78	3.5[b] 0-4	<0.05	—
Bosari et al. (1993)	46 (1801)	Saponin	CM1	206	>5 5-9	<0.02	NS
Sun et al. (1992)	24 (CM1)	—	1801	293	? 1-8	NS	NS
Nathanson et al. (1994)	62 (1801)	ARS[d]	—	84	? 5-10	NS	NS

[a]Target unmasking fluid. [b]Median. [c]No Dukes' stage included in multivariate analysis. [d]Antigen retrieval system.

1991; Purdie et al., 1991; Scott et al., 1991; Hanski et al., 1992; Remvikos et al., 1992; Starzynska et al., 1992; Yamaguchi et al., 1992; Bell et al., 1993; de Angelis et al., 1993; Kaklamanis et al., 1993; Bosari et al., 1994; Nathanson et al., 1994).

Established markers for prognosis such as Dukes' stage and differentiation grade were independently associated with survival, validating this study population. In this long-term follow-up study we found that nuclear p53 protein expression is not related to outcome after surgery. This result is similar to previous studies (Scott et al., 1991; Sun et al., 1992; Bell et al., 1993; Nathanson et al., 1994). Yamaguchi et al. (1992) reported p53 positivity to be of independent prognostic importance, but in their multivariate analysis Dukes' stage was not included. Both Bosari et al. (1994) and Sun et al. (1992) found cytoplasmic p53 staining with PAb CM1 to be of independent prognostic importance, but as in most other studies no reliable cytoplasmic staining was found in our study.

The lack of consistent prognostic value of nuclear p53 protein expression might indicate that the reported importance for prognosis of p53 mutation needs additional study and/or that the relationship between immunostaining of p53 protein and p53 gene mutation might be too much confounded by other biological mechanisms and technical caveats (reviewed in Wynford-Thomas, 1992; Hall and Lane, 1994).

In conclusion, this study indicates that, with current methodology, p53 protein expression does not appear to contribute to the prediction of long-term prognosis after resection of colorectal carcinoma. We emphasise that further insight into the relationship between p53 gene mutations and p53 protein expression is needed to elucidate more precisely their clinical relevance.

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