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Original Paper

Nuclear p53 Protein Expression in Resected Hepatic Metastases from Colorectal Cancer: an Independent Prognostic Factor of Survival

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An association has been reported between nuclear p53 protein expression in tumour cells and a poor outcome in patients with colorectal cancer (CRC). In this study we investigated the prognostic significance of nuclear p53 protein expression in CRC liver metastases after curative hepatic resection. The study population consisted of 69 consecutive patients who underwent curative hepatic resection for metastases from CRC at our Institution between February 1987 and October 1993. Immunohistochemical expression of p53 protein was evaluated in formalin-fixed paraffin-embedded sections of CRC liver metastases using the monoclonal antibodies (MAbs) D01 and Pab 1801. The Cox proportional hazards model was used in forward stepwise regression to assess the relative influence of different prognostic factors. Forty-four (63.8%) CRC liver metastases were p53-positive. Kaplan–Meier survival curves demonstrated that patients with p53-positive metastases had a median survival of 27 months versus 93 months for patients with p53-negative metastases ($P < 0.01$). The 3 and 5 year survival rates were 31.5 and 21.0% in patients with p53-positive metastases and 71.8 and 53.1% in patients with p53-negative metastases. At multivariate analysis p53 protein status was the single best predictor of survival ($P = 0.0079$); the odds ratio of death among patients with p53-positive tumours was 2.53. Nuclear p53 protein expression in hepatic metastases from CRC is an independent prognostic factor of survival following liver resection. These findings may be of clinical importance in the selection of patients more likely to benefit from liver resection and could be used as criteria for stratification in trials on adjuvant therapy.

Key words: p53 protein, colorectal cancer, liver metastases, prognosis
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INTRODUCTION

IN WESTERN countries colorectal cancer (CRC) is the second cause of cancer-related deaths [1]. Up to 25% of patients presenting with primary CRC have synchronous liver metastases and up to 50% of patients will develop metachronous liver metastases after primary treatment [2, 3]. Metastases, confined to the liver in approximately one fourth of all CRC patients, are resectable in approximately 30% of the cases [4, 5].

In natural history studies on patients with untreated CRC liver metastases, median survivals range from 5–8 months, although some long-term survivors are observed [6, 7]. With a median survival of up to 30 months and a 5-year survival of more than 25%, resection appears to be the only potential cure for patients with limited hepatic disease [8–12]. Substantial variations are, however, observed in the long-term survivals of patients with similar conventional prognostic factors, indicating that biological elements may play an important role in determining survival. More accurate markers are therefore required to identify patients at higher risk of recurrence after resection of CRC liver metastases.

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In primary CRC, alterations of specific oncogenes and tumour suppressor genes, appear to be associated with tumour aggressiveness and poorer outcome [13], yet few studies have investigated the prognostic significance of biological variables in CRC liver metastases [14–18].

Mutations of the *p53* tumour suppressor gene are the most common genetic abnormalities to be found in human neoplasms [19]. Such mutations described in about 50% of CRC are thought to be a late event in the multistep process of CRC tumorigenesis [20]. We demonstrated elsewhere that, in CRC, *p53* gene and protein alterations are associated with an increased incidence of regional and distant metastases [21] and there is increasing evidence that these abnormalities may have an adverse prognostic significance [22–35]. Our hypothesis was that nuclear p53 protein expression detected by immunohistochemistry could be of prognostic significance after curative resection of hepatic metastases from CRC and this was examined in this study.

PATIENTS AND METHODS

Patients

The study population consisted of 69 consecutive patients (34 men, 35 women; mean age 53 years, range 22–84 years) who underwent 'curative' hepatic resection for CRC liver metastases at our Institution between February 1987 and October 1993. The primary tumour site was the colon in 50 (72.5%) patients and the rectum in 19 (27.5%) patients. The regional primary tumour lymph nodes were metastatic in 55 (79.7%) cases (Dukes' C lesions). Liver metastases were synchronous in 16 (23.2%) and metachronous in 53 (76.8%) patients. 47 (68.1%) patients had single metastases and 22 (31.9%) multiple: 13 of the latter had two metastases, 8 had three and 1 had six. The mean diameter of the metastases was 4.1 cm (range, 1–13 cm). 30 patients (43.5%) underwent anatomical hepatic resection (8 hemihepatectomy, 10 bisegmentectomy and 12 segmentectomy) and 39 patients (56.5%) underwent wedge hepatic resection. Patients with a disease free resection margin of less than 1 cm (9 patients) and those with more than two metastases (9 patients) were considered at high risk of recurrence and underwent postoperative hepatic artery infusional chemotherapy. 10 (14.5%) patients underwent subsequent liver resection for recurrent disease.

After hepatic resection, patients were followed-up by clinical examination, hepatic enzymes and serum carcinoembryonic antigen (CEA) assay, liver computed tomography (CT) scan or liver ultrasound (US), every three months during the first 2 years following resection, and every 6 months thereafter. Recurrences were confirmed by CT scan or US guided biopsy.

Immunohistochemical analysis

In all samples immunohistochemical staining was performed on formalin-fixed, paraffin embedded tissue using the anti-p53 MAbs, DO1 (Immunotech, Marseille, France) and Pab 1801 (Oncogene Science, Uniodale, U.K.).

Four micron sections were deparaffinised and rehydrated and, as previously described [32], were placed in 10 mM sodium citrate buffer at pH 6 and microwaved for three cycles of 5 minutes each at 750 W, using a Bosch microwave oven. Immunohistochemical staining was subsequently performed using an avidin-biotin peroxidase kit (Vector Laboratories, Burlingam, California, U.S.A.). The Pab 1801 antibody was applied at a dilution of 1/50, while the DO1 antibody was prediluted. 3-3′ diaminobenzidine tetrachloride was used as chromogen and the sections were counterstained with Mayer's hematoxylin. Sections of colorectal carcinoma known to be immunoreactive for p53 were used as positive controls for Pab 1801 and DO1 antibodies. Negative controls were obtained by omitting the primary antibodies. Pab 1801 and DO1 stainings were scored semiquantitatively. Cases with less than 10% p53-positive cells were considered p53-negative.

Statistical analysis

The frequency of p53 positive tumours was assessed for each variable using the Chi-square and Fisher's exact tests where appropriate. Survivals were measured from the date of liver resection until death or last follow-up; data on survival were censored if the patient was still alive at the time of the last follow-up visit. Survival curves were plotted using the Kaplan–Meier method [36] and differences were assessed by the Mantel–Cox and Breslow tests. The Cox proportional hazards [37] model was used in forward stepwise regression to assess the relative influences of the following covariates: age (≤ 55 or > 55 years), sex, p53 protein status (positive versus negative); pre-operative normal versus elevated serum gamma-glutamyl-transpeptidase (GGT); alkaline phosphatase (ALK-P) and serum CEA levels, primary tumour stage (Dukes' A-B versus C); time of liver metastases appearance (synchronous versus metachronous); size (≤ 3 cm versus > 3 cm) and number of liver metastases ($= 1$ versus > 1); percentage of liver involvement ($\leq 30\%$ versus $> 30\%$); type of resection (anatomic versus wedge); disease-free resection margin (> 1 cm), postoperative chemotherapy (yes versus no) and subsequent liver resection for recurrent disease (yes versus no). All statistical analyses were performed using BMDP Statistical Software. *P* values of less than 0.05 were considered statistically significant.

RESULTS

There were no peri-operative deaths and no patients were lost to follow-up. During a median follow-up of 35.6 months, 46 (66.7%) of the 69 patients died of specific disease (there were no deaths from other causes). Median disease-free survival was 17 months; 51 patients had recurrences after liver resection (30 to the liver only, 6 to the lungs only, 6 to the liver and to the lungs, 3 to the liver and peritoneum, 5 to the

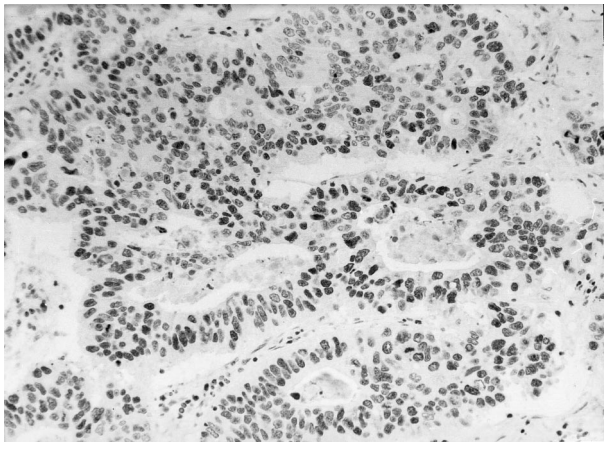

Figure 1. Hepatic metastasis from CRC. Tumour cells exhibiting strong p53 nuclear immunoreactivity (×180).

pelvis and 1 to the brain). Median estimated survival for the entire group of patients was 33 months.

44 of the 69 (63.8%) CRC liver metastases examined were p53-positive (Figure 1); all normal hepatic tissue samples examined for p53 protein expression were negative. No significant differences were observed between the anti-p53 MAb DO1 and Pab 1801 for the intensity and in the pattern of immunostaining. The percentages of tumour cell nuclei staining positive for p53 protein were: greater than 75% in 15 cases; between 25 and 75% in 25 cases; and between 10 and 25% in four cases.

No statistically significant associations were found between the frequency of p53-positive staining and the clinical and pathological characteristics of the patients reported in the Patients and Methods section.

Table 1. *Univariate analysis of survival according to clinical and pathological findings*

Variable	Patient No.	Median Months	Median SEM	Statistical significance
Primary stage				
Dukes' A-B	14	39	8.1	NS
Dukes' C	55	32	7.2	
Time of appearance				
synchronous	16	29	2.0	NS
metachronous	53	35	6.2	
Number of metastases				
= 1	47	35	6.6	NS
> 1	22	29	12.1	
Diameter of metastases				
≤ 3 cm	31	30	4.8	NS
> 3 cm	38	33	9.8	
Liver involvement (%)				
≤ 30	57	35	5.8	NS
> 30	12	27	4.9	
Disease-free resection margin				
≤ 1 cm	9	33	5.4	NS
> 1 cm	60	30	13.0	
p53 protein expression				
negative	25	93	29.1	Mantel–Cox $P = 0.0054$
positive	44	27	2.2	Breslow $P < 0.0128$
Type of resection				
anatomic	30	30	7.8	NS
wedge	39	35	5.5	
Postoperative chemotherapy				
no	51	30	4.4	NS
yes	18	33	8.7	
Re-resection				
no	59	30	4.2	NS
yes	10	39	11.6	
Pre-operative serum CEA*				
≤ 5 ug/L	23	36	10.1	NS
> 5 ug/L	34	30	6.7	
Pre-operative serum GGT*				
≤ 65 U/L	51	36	4.8	NS
> 65 U/L	12	17	2.6	
Pre-operative serum ALK-P*				
≤ 140 U/L	51	35	5.3	NS
> 140 U/L	12	29	15.0	

SEM, standard error of the mean; NS, not significant; CEA, carcinoembryonic antigen; GGT, gamma-glutamyl transpeptidase; ALK-P, alkaline phosphatase. *Some data missing.

Results of univariate analysis of survival are reported in Table 1. The only variable with a significant impact on survival was p53 protein status. Kaplan–Meier survival curves (Figure 2) demonstrated that patients with p53-positive metastases had a median survival of 27 months versus 93 months for patients with p53-negative metastases (Mantel–Cox's test $P = 0.0054$, Breslow's test $P = 0.0128$). During follow-up, 34 (77.3%) patients with p53-positive metastases and 12 (48.0%) with p53-negative metastases died of specific disease. The 3 and 5 year estimated survival rates were 31.5 and 21.0% in patients with p53-positive metastases and 71.8 and 53.1% in patients with p53-negative metastases (Table 2). No significant differences were observed between the two groups of patients for recurrence sites.

At multivariate analysis, the only two variables retained in the model after the Cox proportional hazards model was applied in forward stepwise regression was p53 protein status. The resulting model was significant at $P = 0.006$. The odds ratio of death among patients with p53-positive tumours was 2.53 ($P = 0.0079$) (Table 3).

DISCUSSION

Although findings reported are not in complete agreement, factors that seem associated with a better prognosis following hepatic resection of colorectal metastases are, disease-free

Table 2. *Estimated survival rates in patients with p53-negative and p53-positive metastases*

p53 Protein expression	Survival 2-year (%)	3-year (%)	4-year (%)	5-year (%)
p53-negative ($n = 25$)	76.0	71.8	53.1	53.1
p53-positive ($n = 44$)	56.8	31.5	31.5	21.0

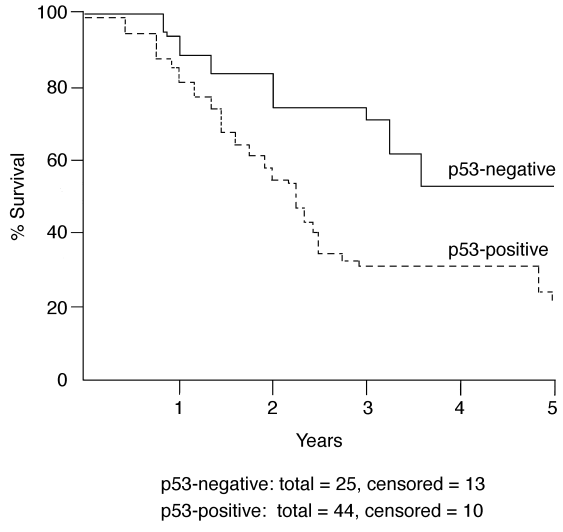

Figure 2. Kaplan–Meier survival curves of 69 patients resected for hepatic metastases from CRC in relation to p53 protein expression. The difference between the two curves was statistically significant (Mantel–Cox's test $P = 0.0054$; Breslow's test $P = 0.0128$).

Table 3. *Multivariate analysis of survival**

Variable	Category	Hazard ratio	95% CI	*P* value
Nuclear p53 protein expression	p53-positive versus p53-negative	2.53	1.84–3.22	0.0079

Final model after using the Cox proportional hazards model in a forward stepwise regression. *Variables entered in multivariate analysis are reported in the statistical analysis section.

survival, interval after primary tumour resection and hepatic resection margins [8, 38, 39]. Other more controversial factors include, primary tumour stage, pre-operative CEA values, number of metastases and type of liver resection performed [8, 39–41]. In a recent study patients with metastases from colorectal carcinoma were, on the basis of the more important prognostic factors, subdivided into three risk groups with different 2-year survival rates and a simple prognostic scoring system was proposed to evaluate the chances of cure following hepatic resection [12].

The existence of long term survivors among patients with unresected CRC liver metastases together with the variable outcome of patients with similar conventional prognostic factors after resection of CRC liver metastases suggest that biological tumoral characteristics may play an important role in determining long-term survival.

As yet, few studies have focused on the identification of biological tumoral factors affecting survival after resection of CRC liver metastases. In a group of 51 patients, Cady and associates found a close correlation between the diploid pattern on flow cytometry of liver metastases from CRC and disease-free survival after resection [15] and in a group of 35 patients, Yamaguchi and colleagues reported a significantly longer survival in those with diploid than in those with aneuploid metastases [13]. In their group of 37 patients, however, Lind and coworkers found no difference between the survival of patients with diploid and that of those with aneuploid metastases [14]. In a group of 75 patients, Silvestrini and associates reported a threefold risk of death in patients with high [3H] thymidine labelling index metastases [16]. In a more recent study on a group of 33 patients, Kastrinakis and colleagues found that neither the presence of K-*ras* mutations in metastases nor the precise nucleotide change were predictive of long-term survival [17].

Several authors have described p53 protein expression in 39–67% of CRCs [21, 26–35] and several studies have reported that p53 positivity is associated with advanced stages [21, 26–30, 35]. In the present study, in agreement with the above authors, we found a high incidence (63.8%) of metastases expressing p53 immunoreactivity, thus confirming that *p53* gene alterations are late events in the multistep process of CRC tumorigenesis.

The results of our multivariate analysis of survival showed that nuclear p53 protein expression in metastases is independently associated with shorter survival. This is in line with findings made in several other studies that nuclear p53 protein expression in CRC has an adverse prognostic significance, especially in more advanced stages [26–35].

The *p53* gene plays a central role in the control of many critical processes related to tumour development and progression. In fact, the *p53* gene is involved in the induction of apoptosis reducing *Bcl2* expression and acting as a transcriptor factor for *Bax* [42]. Moreover, *p53* controls the DNA repair mechanisms by arresting cells in late G1 phase and stimulating the DNA repair machinery [43]. Finally, wild-type *p53* seems to inhibit angiogenesis by stimulating the thrombospondin 1 gene [44]. Wild-type p53 protein has a very short half-life and cannot usually be detected by immunohistochemistry. Changes in its conformation due to mutations appear to stabilise the protein, leading to nuclear accumulation, with positive immunostaining. Other processes (e.g. interaction of the protein with other nuclear cellular and viral proteins or an increase in wild-type p53 protein levels in response to continuous DNA damage) can lead to the accumulation of nuclear p53 protein [45, 46]. Consequently, the immunohistochemical detection of nuclear p53 protein seems to indicate an altered *p53* gene functional status, although molecular analysis is required for the identification of specific *p53* gene alterations. In CRC, however, there is a concordance of approximately 70% between p53 protein expression detected by immunohistochemistry and the presence of abnormalities within the *p53* gene as detected by molecular analysis [47–49].

Since the immunohistochemical detection of nuclear p53 protein indicates that the protein is mutated or inactivated, the more aggressive behaviour of tumours overexpressing p53 protein may be explained by the loss of *p53* gene functions. Of course more specific molecular studies are required to validate this hypothesis.

If its apparent impact on survival is confirmed, nuclear p53 protein status may be of clinical utility in the selection of patients more likely to benefit from liver resection. In addition, p53 protein status could be considered in the stratification of patients prior to randomisation in future prospective studies comparing hepatic resection alone versus hepatic resection plus systemic and/or locoregional adjuvant chemotherapy.

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