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p53 and Ki-*ras* as prognostic factors for Dukes' stage B colorectal cancer

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Abstract

Mutations of the *TP53* and Ki-*ras* genes have been reported to be of prognostic importance in colorectal carcinomas. An increased intracellular concentration of the p53 protein, although not identical to, is sometimes seen in tumours with *TP53* mutation and has been correlated with poor prognosis in some tumour types. Previous colorectal cancer studies, addressing the prognostic importance of Ki-*ras* mutation and *TP53* aberrations, yielded contradictory results. The aim of this study was to determine in a clinically and therapeutically homogeneous group of 122 sporadic Dukes' B colorectal carcinomas with a median follow-up of 67 months (3–144 months) whether or not p53 protein expression, *TP53* mutation and K-*ras* mutation correlated with prognosis. p53 staining was performed by immunohistochemistry, using the monoclonal antibody DO7 on paraffin-embedded tissue. Mutations in exons 5–8 of the *TP53* gene and in codons 12 and 13 of the K-*ras* gene were assayed in paraffin-embedded tissue by the single-strand conformation polymorphism (SSCP) assay. Nuclear p53 staining was found in 57 (47%) tumours. Aberrant migration patterns indicating mutation of the *TP53* gene were found in 39 (32%) tumours. Forty-six carcinomas (38%) showed a mutation of the Ki-*ras* codons 12 or 13. In a univariate analysis, patients with wild-type *TP53* status showed a trend towards better survival, compared with those with mutated *TP53* (log-rank test, $P=0.051$). Likewise, tumours immunohistochemically positive for p53 showed a worse prognosis than p53-negative tumours ($P=0.010$). The presence or absence of mutations in Ki-*ras* did not correlate with prognosis ($P=0.703$). In multivariate analysis, only p53 immunoreactivity emerged as an independent marker for prognosis hazard ratio (HR) = 2.16, 95% confidence interval (CI) 1.12–4.11, $P=0.02$). Assessment of p53 protein expression is more discriminative than *TP53* mutation to predict the outcome of Dukes' stage B tumours and could be a useful tool to identify patients who might benefit from adjuvant therapy.

Keywords: Dukes' B; Colorectal carcinoma; p53 expression; *TP53* mutation; Survival

1. Introduction

The clinical behaviour of colorectal cancer is highly variable. Amongst various clinicopathological parameters such as histological degree of differentiation, vascular invasion, mucin production, size and location of the tumour, tumour stage is the most significant prognostic indicator [1]. However, there is important heterogeneity within a single stage category since approximately 1/3 of patients with Dukes' stage B and 2/3 of patients with Dukes' stage C will die of recurrent disease [2]. Approximately one-third of all colorectal tumours are Dukes' stage B and as yet, there are no parameters available allowing the subdivision of this group of patients into more accurate prognostic strata and no marker to reliably predict which patients are likely to benefit from adjuvant therapy.

It is now widely accepted that colonic carcinogenesis is a multistep process in which multiple mutational events, including activation of Ki-*ras*, inactivation of adenomatous polyposis coli (*APC*), deleted in colorectal cancer (*DCC*), *TP53* and inactivation of DNA mismatch repair genes are implicated.

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Ki-*ras* mutations occur early in colorectal carcinogenesis, mostly before adenoma formation, whereas *TP53* mutations are implicated in the progression from adenoma to carcinoma. In colorectal cancers, the *TP53* gene often shows missense mutations in one allele, accompanied by loss of the wild-type allele [3]. These missense mutations result in mutant p53 proteins with a prolonged half-life, which renders them detectable by immunohistochemical analysis [3–5]. For as much as mutational events are the driving force in the process of colonic carcinogenesis, the hypothesis that the profile of mutations in a particular cancer would predict its behaviour seems justified. However, since the reported results of such studies are very contradictory, the impact of cancer associated gene mutations on the prognosis of colorectal carcinoma is still unresolved. Indeed, some studies showed that *TP53* aberrations and Ki-*ras* mutations were not correlated with prognosis [6] whereas other studies indicated that mutations of these genes correlated with poor prognosis [7–10]. In contrast, Dix and colleagues observed that patients whose tumours showed p53 protein expression by immunohistochemistry had a significantly better prognosis than those whose tumours had no p53 immunoreactivity [4]. Part of this controversy might stem from the fact that in most reported series rather heterogeneous patient groups were studied. We, therefore, examined a large retrospective but clinically and therapeutically homogeneous, series of primary Dukes' stage B colorectal carcinomas, addressing the question of whether Ki-*ras* mutations and/or *TP53* aberrations, as detected by molecular genetic analysis and immunohistochemistry, have any prognostic value in this group of patients.

2. Patients and methods

2.1. Patients

Patients who underwent curative surgical treatment performed between January 1985 and August 1989 at the Centre Hospitalier Universitaire Vaudois, Lausanne, for primary sporadic Dukes' stage B colorectal carcinoma, were enrolled in this study. Cases of inherited non-polyposis colorectal cancer, familial adenomatous polyposis or ulcerative colitis and patients who died in the immediate postoperative period were excluded from the study. Selected patient characteristics are presented in Table 1. A total of 122 cases (65 men and 57 women; median age: 71 years; range: 37–90) was collected. All patients included in this study underwent a colectomy performed by the same team of surgeons, without pre- or postoperative adjuvant therapy and the group can thus be considered to be therapeutically homogeneous. The tumour stage was based on clinical evaluation including pre-operative chest X-ray, abdominal ultrasound or computed tomography (CT) scan and abdominal exploration during laparotomy. Tumours infiltrating the wall of the colon beyond the muscularis propria without metastasis were considered as stage B, according to Dukes' original classification [11].

Table 1
Relationship between p53 protein expression, *TP53* and Ki-*ras* mutations, and clinicopathological variables in Dukes' stage B colorectal cancers

	Number of patients *n* (%)	p53 expression *n* (% positive)	*P* value	*TP53* mutation *n* (% positive)	*P* value	Ki-*ras* mutation *n* (% positive)	*P* value
All patients	122 (100)	57 (47)	–	39 (32)	–	46 (38)	–
Sex							
Female	57 (47)	20 (35)	0.016	20 (35)	0.489	19 (33)	0.351
Male	65 (53)	37 (57)		19 (29)		51 (42)	
Age (years)							
< 70	52 (43)	23 (44)	0.635	14 (27)	0.303	22 (42)	0.366
> 70	70 (57)	34 (49)		25 (36)		24 (34)	
Tumour location							
Proximal	38 (31)	8 (21)	< 0.01	6 (16)	0.034	13 (34)	0.511
Distal	49 (40)	27 (55)		20 (41)		17 (35)	
Rectum	35 (29)	22 (63)		13 (37)		16 (46)	
Tumour differentiation							
Well	17 (14)	7 (41)	0.024	5 (29)	0.662	5 (29)	0.513
Moderate	91 (75)	48 (53)		31 (34)		37 (41)	
Poor	14 (11)	2 (14)		3 (21)		4 (29)	
Mucinous component							
Yes	33 (27)	5 (15)	< 0.01	6 (18)	0.047	16 (49)	0.135
No	89 (73)	52 (58)		34 (38)		30 (34)	

Tumours were histologically classified as well differentiated (14%), moderately differentiated (75%) or poorly differentiated (11%) adenocarcinomas using the WHO criteria [12]. A partial or predominant mucinous adenocarcinomatous component was observed in 27% of cases. Thirty-eight tumours (31%) were localised in the proximal colon and 84 tumours (69%) were in the distal colon or in the rectum.

After surgery, the patients were entered into a follow-up programme, which included a combination of the following parameters: serum carcinoembryonic antigen (CEA) level which was assessed every 3 months during the first 2 years and every 6 months thereafter endoscopic procedures, ultrasonography or CT scan and chest X-ray.

Follow-up was available for all patients at the date set for collecting data, April 1994. Thus, overall survival was considered as the endpoint for evaluating the prognostic significance of the variables collected. The median follow-up was 67 months (3–144 months). Of the 122 patients, 39 (32%) died of colorectal cancer, 33 (27%) within 5 years. One patient died of unrelated disease.

2.2. Samples

Slides of 122 cases were reviewed by three pathologists. For every case, one paraffin block with both tumour tissue and normal mucosa was selected for the detection of p53 protein expression by immunohistochemistry.

For Ki-*ras* and *TP53* mutation analysis, DNA was extracted from paraffin-embedded tissue. Based on the assumption that colorectal adenocarcinomas may be genetically heterogeneous, two different tumour samples were analysed in each case. When genetic heterogeneity was found for *TP53* mutation both samples were analysed for p53 expression by immunohistochemistry.

2.3. Immunohistochemistry

Four micrometre thick tissue sections were mounted on aminopropylmethoxysilane-coated glass slides, deparaffinised in xylol, blocked for endogenous peroxidase with 1% H_2O_2 in methanol (45 min) and rehydrated with graded alcohols. Samples were then subjected to microwave oven heating for 15 min in 10 mM citrate buffer pH 6.0 and rinsed in TBS (Tris 0.05 M, NaCl 0.9%, pH 7.6). In order to reduce non-specific binding, they were incubated for 10 min in normal goat serum (Pel-Freez Biologicals, Rogers, AK, USA) 1: 30 in TBS. After incubation (30 min) with the primary monoclonal antibody (mouse anti-p53, clone DO-7, Dako, Glostrup, Denmark) diluted 1:500 in TBS containing 5% non-fat dry milk (TBS-nfdm), the sections were incubated (30 min) with goat anti-mouse immunoglobulins (Sternberger, Baltimore, MD, USA) diluted 1: 100 in TBS-nfdm, and with PAP-complex diluted 1: 600 in TBS-nfdm. Peroxidase activity was revealed with 5-5, diaminobenzidine as the chromogen and the sections were counterstained in Mayer's acid-free haematoxylin. As a negative control, the first-step monoclonal antibody was replaced by a hybridoma supernatant of similar isotype without reactivity for the tissue examined.

Immunoreactivity for p53 was evaluated semi-quantitatively by three observers and, according to the percentage of positive tumour nuclei, scored as follows: (0) for tumours showing less than 10% of immunostained nuclei, (+) for tumours showing 10–50% of immunoreactive nuclei; (++) for those tumours with nuclear immunoreactivity in more than 50% of tumour cells.

The observers examined the slides independently and were blinded to outcome. There was a complete concordance between the observers for the presence of p53 nuclear staining. Those tumours for which there was some interobserver variation in the determination of the percentage of p53-positive cells were reassessed on a multiheaded microscope by the three pathologists.

2.4. Detection of *Ki*-ras *and* TP53 *mutations by non-radioactive single strand conformation polymorphisms (SSCP)*

Tumour tissue was dissected from paraffin blocks with a sterile scalpel. After deparaffinisation in xylene and proteinase K digestion, the DNA was extracted with phenol and phenol/chloroform and precipitated with ethanol [13]. Ki-*ras* exons 1 and 2 and *TP53* exons 5 to 8 were amplified separately by polymerase chain reaction (PCR). The PCR reaction was carried out for 35 cycles using the following amplification profile: denaturation at 94°C for 30 s, annealing at 54–60°C for 45 s, and extension at 73°C for 45 s. Correct amplification was controlled by electrophoresis on a 2% agarose gel.

Five to forty nanograms of PCR product were denatured in 10 μl of 50 mM NaOH and 1 mM EDTA at 50°C for 10 min. These conditions allow an almost complete denaturation of the DNA. After addition of 1.5 μl of formamide-dye, the samples were immediately analysed in a 12% MDE gel (FMC BioProducts, Rockland, ME, USA). Electrophoresis was performed at 20°C on a vertical gel in a Hoeffer SE600 apparatus, at 20 V/cm in 0.5×TBE for approximately 4 h [14]. The gels were stained with SYBY green II (FMC BioProducts) and visualised under ultraviolet (UV) light using a CCD camera. This technique allows the detection of at least 10 mutated alleles amongst 100 wild-type alleles. Primers for exons 5–8 of the *TP53* gene have previously been described [15]. Exons 1 and 2 of the Ki-*ras* gene

were amplified by the following primers: Ras-ex1-5′: GACTGAATATAAACTTGTGG and Ras-ex1-3′: TCCTGGTCCTGCACCAGTAAT for exon 1; Ras-ex2-5′: GACTGTGTTTCTCCCTTCT and Ras-ex2-3′: TGGCAAATACACAAAGAAAG for exon 2.

2.5. Statistical analysis

The primary endpoint for this analysis was overall survival (OS), defined as time from surgery to death. Statistical analyses were carried out by means of the software package Stata [16]. Proportions were compared using Chi-square tests. Survival percentages over time were calculated by the Kaplan–Meier method and their corresponding standard errors of the mean (SEM) with Greenwood's formula. For univariate analyses, the P values of the log-rank test are reported. Estimated hazard ratios (HR) of death (or of first event), with respect to the chosen reference group, their 95% confidence intervals (95% CI) and P values were calculated with a multivariate Cox proportional hazard regression model: appropriate binary variables were generated to identify each subgroup of interest. Values of HR greater than unity indicate increased rates of death with respect to the chosen reference category. The prognostic factors used in the survival analysis were as follows: the age of the patients ($\geqslant 70$ versus < 70 years), gender, location of the tumour (proximal versus distal and rectal colon), histological grade (poorly and moderately versus well differentiated), presence or not of mucinous component, p53 protein overexpression (0 versus (+) or (++)) , *TP53* mutation (absent versus present), Ki-*ras* mutation (absent versus present). Forward selection was used to build the final model. All reported probability values are for two-sided tests.

3. Results

3.1. p53 analysis

Immunohistochemical staining for p53 was carried out on a total of 122 cases. Normal mucosa samples were invariably negative for p53. p53 expression was detected in 57 (47%) colorectal adenocarcinomas (Table 1). The immunoreactivity was confined to the nuclei of neoplastic cells. Out of these 57 cases, 42 (74%) displayed a diffusely positive (++) pattern of immunoreactivity with more than 50% of positive tumour cells whereas 15 (26%) showed a partially positive (+) pattern where only 10–50% of cancer cells were stained. A *TP53* mutation was found in 39 (32%) tumours, of which 30 (25%) also expressed the p53 protein by immunohistochemistry. Interestingly, 5 cases were heterogeneous for the *TP53* mutation since only one of the two samples of carcinoma tested by SSCP was positive. When these 5 cases were tested for p53 expression in both tumour blocks, 4/5 presented the same p53 expression in the two blocks whilst the other case showed an absence of p53 in the first block and a presence in the second block thereby illustrating the differences between immunohistochemical and genetic analyses.

3.2. Ki-ras analysis

Of the 122 colorectal cancers analysed for Ki-*ras* mutations at codons 12 and 13, 46 (38%) showed a mutation. A mutation of codon 12 alone was observed in 33 (72%) colorectal carcinomas and of codon 13 alone in 11 (24%) colorectal carcinomas. In 2 cases, we found a mutation in both codons 12 and 13. The most frequent mutations at codon 12 were GAT and GTT. In 17 patients (14%) with a Ki-*ras* mutation, p53 protein was also detected by immunohistochemistry and 11 (9%) other Ki-*ras*-mutated patients also had a *TP53* mutation. 5 Ki-*ras* mutated tumours showed both *p53* overexpression and *TP53* mutation whilst 13 showed mutation in Ki-*ras* alone. Ki-*ras* mutation heterogeneity was found in 6 cases (only one of two sample analysed mutated), one of these cases showed both *TP53* and Ki-*ras* mutation heterogeneity. No further mutational analysis was carried out for Ki-*ras* as our previous studies have shown that mutations detected and identified by SSCP analysis were always confirmed upon further allelic-specific PCR [17].

3.3. Statistical subgroup analysis

Table 1 summarises the correlation between p53 expression, *TP53* mutation and Ki-*ras* mutation and the clinicopathological features. A statistically significant correlation emerged between p53 expression and *TP53* mutation and distal/rectal tumour location ($P < 0.01$ and $P = 0.034$, respectively), and absence of a mucinous component ($P < 0.01$ and $P = 0.047$, respectively). Otherwise, well differentiated tumours were inversely correlated with the expression of p53 protein ($P = 0.024$). The combination of *TP53* abnormalities and Ki-*ras* mutations did not define a subgroup with distinct clinicopathological characteristics. There was no correlation between Ki-*ras* mutations at codons 12 and 13 analysed together and any of the clinicopathological variables.

3.4. Survival analysis

The 5-year survival rates by marker status are shown in Table 2. A comparison of our results with those of previous studies is shown in Table 3. Patients with p53 protein expression (+ and ++ taken together) showed a worse prognosis than patients without p53 expression (log-rank test, $P = 0.010$). There was no difference in

Table 2
Survival rates and marker status in patients with Dukes' stage B colorectal cancer

Markers	No. of patients *n* (%)	No. of deaths *n* (%)	5-year % survival ± SEM	log-rank *P* value
p53 expression				
Positive > 10%	57 (47)	25 (44)	63 ± 6	0.010
Negative	65 (53)	15 (23)	78 ± 5	
TP53 mutation				
Positive	39 (32)	17 (44)	64 ± 7	0.051
Negative	83 (68)	23 (28)	78 ± 5	
Ki-*ras* mutation				
Positive	46 (38)	17 (37)	71 ± 7	0.703
Negative	76 (62)	23 (30)	73 ± 5	

survival between patients with low to moderate (+) or high (++) p53 protein expression (Fig. 1). Patients with a tumour with wild-type *TP53* showed a better survival than those harbouring a *TP53* mutation (log-rank test, $P = 0.051$). Neither the Ki-*ras* status (wild-type versus mutated) nor the combination of Ki-*ras* and *TP53* status correlated with prognosis.

When modelling survival by means of a multivariate Cox regression model, only age (below or above 70 years) and p53 overexpression achieved statistical significance. Older patients were at higher risk of death (HR = 2.11, 95% CI 1.05–4.24, $P = 0.03$) as well as patients with >10% p53-positive tumour cells (HR = 2.16, 95% CI 1.12–4.11, $P = 0.02$). The other markers or other clinical and demographic variables did not correlate significantly with survival.

4. Discussion

Amongst the genetic abnormalities implicated in the development of colorectal cancer Ki-*ras* and *TP53* mutations have probably been the most exhaustively studied [26]. Several studies reported that abnormalities of Ki-*ras* and/or *TP53* genes correlate with a worse prognosis [7–9,19,22,23]. However, important discrepancies concerning the correlation of these parameters with the survival of colorectal carcinoma patients have emerged from the literature [6,8,20] with some studies showing no prognostic significance of p53 abnormalities [6] whilst others report p53 expression correlates with poor [7–9] or, conversely, with better prognosis [4].

Likewise, conflicting results have been reported in previous studies on the prognostic significance of Ki-*ras* mutation in colon cancer; some reporting an improved survival in patients with Ki-*ras* mutated tumours [9,10,27–29] and others no correlation at all [6,30]. Several factors should be taken into consideration as an explanation for these contradictory results. A major difficulty in the interpretation of literature data is the use of different staging systems or small groups of different stages. Moreover, the details of patient therapy are often inadequate in prognostic marker studies [31]. Many published studies include patients with all Dukes' stages. A study including patients of all stages obviates the need for substratification and is, therefore, more appropriate for the assessment of the prognostic utility of a marker. The choice of the patient group is also determined by the questions asked, as markers would have little potential clinical use in stage A disease, whereas predictors of tumour response to therapy are

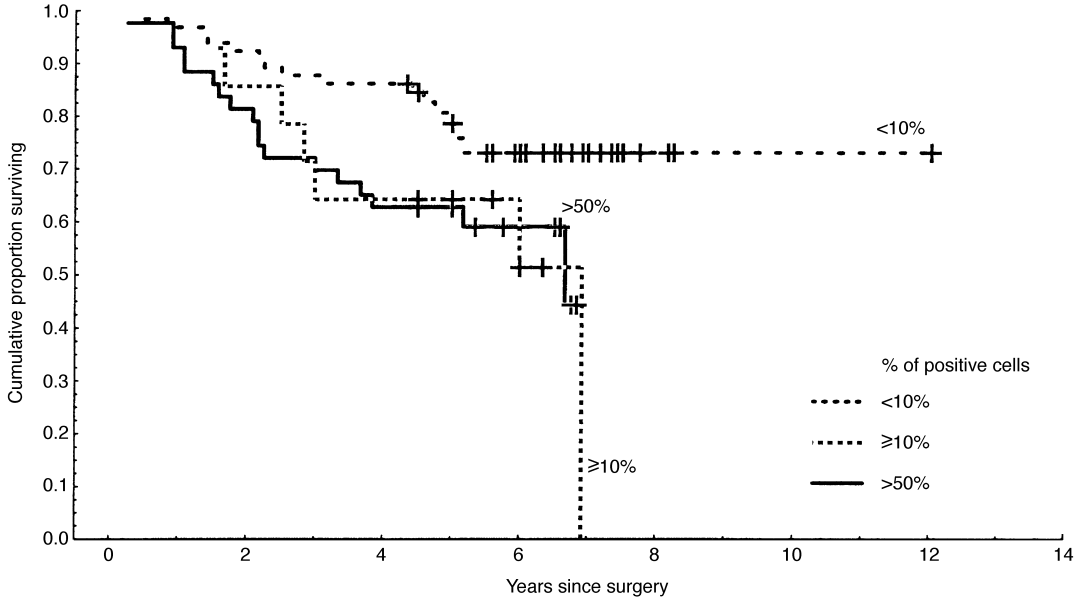

Fig. 1. Survival according to positivity in p53 immunohistochemical expression in Dukes' stage B colorectal carcinomas + censored observations.

Table 3
Comparison of Ki-*ras* mutations and *TP53* mutation/ overexpression and correlation with survival in this and previous studies

Authors	Colorectal cancer *n*	m Ki-*ras* %	m p53 %	p53 exp %	m p53 + m Ki-*ras* %	p53 exp + m Ki-*ras* %	m Ki-*ras* Survival	m p53 Survival	p53 exp Survival	p53 exp + m Ki-*ras* Survival
Shaw [18]	24 Dukes' A–C'[a]	48	64	–	45	–	–	–	–	–
Laurent-Puig [19]	22 Dukes' A									
	39 Dukes' B	37	–	–		–	NS	0.004	–	–
	48 Dukes' C–D	(Dukes' NA)								
Bell [20]	4 Dukes' A	50 Dukes' A		25 Dukes' A		0 Dukes' A				
	50 Dukes' B	18 Dukes' B	–	44 Dukes' B	–	6 Dukes' B	NS	–	NS	Dukes' B + C
	46 Dukes' C	28 Dukes' C		48 Dukes' C		15 Dukes' C				$P < 0.004$
Morrin [21]	52 Dukes' A,B,C	36 Dukes' A,B,C	–	62 Dukes' A,B,C	–	27 Dukes' A,B,C	NS	NS	NS	–
	23 Dukes' B	35 Dukes' B		78 Dukes' B						
Tanaka [22]	39 Dukes' A,B,C	27	–	64	–	–	$P < 0.06$	–	NS	–
Leahy [8]	12 Dukes' A		14 Dukes' A	15 Dukes' A				Dukes' A & B		
	11 Dukes' B	–	50 Dukes' B	12 Dukes' B	–	–	–	$P = 0.01$	0.016	–
	16 Dukes' C		47 Dukes' C	24 Dukes' C				Dukes' C = NS		
Bennett [6]	168 Dukes' B	29	–	48	–	–	NS	–	NS	–
Pricolo [23]	71 Dukes' B		61 Dukes' B	–	–	–	–	Dukes' B $P = 0.02$	–	–
	70 Dukes' C	–	61 Dukes' C	–	–			Dukes' C $P = 0.006$		
Fung [24]	31 Dukes' A	16 Dukes' A								
	87 Dukes' B	24 Dukes' B	–	–	–	–	NS	–	–	–
	74 Dukes' C	27 Dukes' C								
Pauly [25]	72 Dukes' A	1 Dukes' A	4 Dukes' A							
	72 Dukes' B	3 Dukes' B	11 Dukes' B							
	72 Dukes' C–D	4 Dukes' C–D	7 Dukes' C–D	–	–	–	–	–	–	–
			6 Dukes' NA							
Ahnen [9]	66 Dukes' B	43 Dukes' B		56 Dukes' B			Dukes' B $P = 0.003$	–	Dukes' B	
	163 Dukes' C	40 Dukes' C	–	67 Dukes' C	–	–	Dukes' C NS	–	Dukes' C	
									$P = 0.003$	
Bouzourene (this study)	122 Dukes' B	38	32	47	9	14	NS	0.051	0.010	NS

m p53, mutated *TP53*; m Ki-*ras*, mutated Ki-*ras*; p53 exp, p53 overexpression; NS, non significant; NA, not available.
[a] Not specified.

more likely to be found in a group of Dukes' stage C or D patients.

Our series of Dukes' stage B colorectal cancers is of interest as it comprises an important number of cases with long follow-up, and uniform therapeutical approach since all the patients were operated on in the same centre of surgery by the same team of surgeons and no patients received pre- or postoperative chemo- or radiotherapy.

A source of discrepancy concerning *TP53* abnormalities and prognosis of colorectal carcinoma is related to the differences between immunohistochemical and molecular genetic analysis of p53 [7,20,29,32]. Mutations are mostly missense, leading to a protein with a prolonged half life which makes it immunohistochemically detectable [33] in 50–80% of cases [5,34–36]. In addition, viral infection or transcriptional activation subsequent to DNA damage stabilises the p53 protein or increases its expression, thereby making it detectable by immunohistochemistry [36,37]. Inhibition of the proteasome degradation of p53 due to its inability to bind MDM2 has emerged as a third mechanism of p53 accumulation [38]. We, therefore, decided to study both p53 protein expression and *TP53* gene mutation. In agreement with earlier studies [6,7,9,20–22], we observed p53 protein overexpression in 47% of cases, without any correlation with other clinicopathological features, except tumour site and the presence of a non-mucinous component. Mutation of the *TP53* tumour suppressor gene was observed in 32% of cases.

Univariate analysis showed that the survival of patients with immunohistochemically p53-positive tumours or *TP53* mutated cancer was significantly shorter. However, in the multivariate analysis, only p53 expression emerged as an independent marker for prognosis. These results suggest that, for identifying patients at high risk of recurrence and death, immunohistochemical detection of p53 protein may be more useful than SSCP analysis of the *TP53* gene. However, there is no consensus in the literature concerning this point, Pricolo and colleagues suggest routine *TP53* mutation analysis for prognostic purposes in Dukes' B cases [20] whilst Bennett and associates did not find any prognostic impact for p53 overexpression [6]. It should be noted, however, that this study was performed using a different antibody (CM1), which resulted in significant cytoplasmic staining. To avoid such complications we chose, as in the study by Leahy and colleagues [8], to use the DO-7 antibody.

In 38% of tumours, we found a mutation and colleagues in codons 12 and 13 of the Ki-*ras* oncogene but this did not correlate with survival. This frequency is within the range reported by others for Dukes' B stage [6,9,10,18,19,21,22,24,26,27]. Our result confirms those of one of the largest studies of Dukes' B colorectal cancer in which it was found that Ki-*ras* mutation does not correlate with survival [6]. This result, however, is in contradiction with our own previously reported series which showed a prognostic significance of Ki-*ras* mutations in a cohort including Dukes' A B, and C colorectal cancer patients [27] — although, the Dukes' B group in that series was small which might have introduced a selection bias. Our result is also in disagreement with those of Ahnen and colleagues [9] who found Ki-*ras* mutation to correlate with poor prognosis in Dukes' B but not in Dukes' C stage. Also, the collaborative RASCAL study reported a correlation between Ki-*ras* mutation and worse prognosis, multivariate analysis suggesting that the presence of any mutation in Ki-*ras* increases the risk of recurrence and death by 25%, (95% CI 10–42%) independent of the Dukes' stage [10].

In contrast to our results, a significant difference in survival was identified by Bell and colleagues in patients with tumour where both the Ki-*ras* and *TP53* genes were mutated [20]. However, the group of patients with these two oncogenic abnormalities was too small to reach any conclusions with a high degree of certainty.

McLeod and colleagues focused on the divergent results in published reports on the prognostic value of markers in colorectal cancer [31]. They found that negative results were obtained for *ras* mutations when the study was restricted to a specific disease stage (Dukes' B [6]; Dukes' B/C [4]; stage III: [23], whereas those with positive results frequently included a mix of Dukes' A–D tumours.

The literature is not entirely clear on genetic heterogeneity in colorectal neoplasms. Some authors reported this [17,39] whilst others did not [40–42]. Close scrutiny of the published reports reveals that only rarely were multiple samples taken systematically for molecular analysis. Our own study unambiguously confirmed Ki-*ras* mutational heterogeneity in colorectal cancer.

In conclusion, in this relatively homogeneous group of Dukes' B stage colorectal carcinoma patients only p53 overexpression remained an independent molecular marker of unfavourable prognosis.

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