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Prognostic significance of proliferative activity, DNA-ploidy, *p53* and *Ki-ras* point mutations in colorectal liver metastases

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Abstract. Paired colorectal liver metastases (CLM) and normal tissue samples from a consecutive series of 36 patients were studied prospectively. MIB-1 expression was studied by immunohistochemistry on paraffin-embedded sections. DNA ploidy and S-phase fraction (SPF) measurements were performed by flow cytometry on frozen tissues. Mutations within the *p53* (exons 5-8) and c-*Ki-ras* (codons 12 and 13) genes were detected by PCR single-strand conformation polymorphism analysis followed by sequencing. A high correlation was observed between the MIB-1 LI and SPF value (rho=0.81; $P<0.01$). Moreover, *p53* gene mutations were associated with either high MIB-1 LI and high SPF. In univariate analysis, SPF and MIB-1 levels were related to risk of death. The association between overall survival and DNA-ploidy or *p53* mutations did not reach statistical significance, but a slightly better survival was observed for patients either with DNA-diploid tumours or without mutations ($P=0.05$ and $P=0.06$, respectively). SPF was shown by multivariate Cox model analysis to be an independent prognostic variable and thus it might be a useful prognostic factor in patients with CLM.

It is well known that patients with colorectal cancer are at high risk of developing synchronous and/or metachronous liver metastases, with 10% to 25% of them having overt metastases at the time of presentation and up to 40% eventually developing this pathological event (Daly & Kemeny 1997). Though liver metastasis is generally considered a predictive indicator of severe prognosis, clinical course and survival may vary widely on account of different responses to therapy from patient to patient. Moreover, colorectal liver metastases (CLM) also show extremely heterogeneous biological behaviour and there is still some discussion regarding the exact role of biological factors in predicting survival after treatment (Cady *et al.* 1992). The most investigated prognostic indicators in colorectal carcinomas are proliferation associated markers, DNA content and genetic alterations (e.g. *Ki-ras* and *p53* gene mutations) (Gilliland *et al.* 1996, Goh & Smith 1995, Smith *et al.* 1996). Proliferation bioindicators, which may prove to be a measure for predicting biological aggressiveness, have

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been considered good indicators of clinical outcome in many types of cancer (Silvestrini 1994). However, there is still controversy about the best method for measurement of cell kinetic activity (Silvestrini 1994, Gillinand *et al.* 1996). Flow cytometric S-phase fraction (SPF) is a rapid, simple, highly reproducible technique provided that a standardized quality control protocol and the same cell cycle analysis model are used (Silvestrini *et al.* 1994b, Bergers *et al.* 1995, Bergers *et al.* 1996). Furthermore it is, applicable to fresh, frozen or even fixed, paraffin-embedded tissues and its prognostic significance has been extensively investigated, over the last decade in colorectal carcinomas (Bauer *et al.* 1993). Recently, a new monoclonal antibody, MIB-1, raised against recombinant parts of the Ki-67 antigen (Key *et al.* 1992), has been used to evaluate the fraction of proliferating cells on microwave-processed, formalin-fixed, paraffin-embedded tissues. In several tumours, MIB-1 immunoreactivity has been reported to identify the aggressive potential of the tumours and to provide a guide for the evaluation of their proliferation rate (Youssef *et al.* 1995, Pinder *et al.* 1995). A second type of biological variable is the genetic instability as assessed by flow cytometric analysis of DNA content. Generally, DNA-aneuploid tumours are associated with worse prognosis (Bauer *et al.* 1993). In addition, multiple genetic alterations involving both oncogenes and tumour-suppressor genes occur in tumour progression and may affect the biological behaviour of human tumours. Among these, *Ki-ras* and *p53* gene point mutations, the most common genetic changes in a variety of neoplasms (Bos 1989, Nigro *et al.* 1989), have been shown to be associated with dissemination of the colorectal carcinoma and poor prognosis (Hamelin *et al.* 1994, Goh & Smith 1995, Pricolo *et al.* 1996, Smith *et al.* 1996). Although many investigations have focused on the analysis of primary colorectal carcinomas to identify new factors that could be useful in predicting clinical behaviour, cell kinetic and genetic alterations occurring in colorectal liver metastases have not been extensively studied.

We have carried out a prospective evaluation of the MIB-1 index, SPF, DNA-ploidy, *p53* and *Ki-ras* mutations in CLM from a consecutive series of 36 patients. The main objectives of the present study were, first, to analyse the association between biomolecular parameters and, second, to evaluate whether the biological indicators were of greater prognostic significance than traditional clinicopathological variables, such as resectability.

MATERIALS AND METHODS

Patient features paired tumour and normal tissue samples were obtained from a consecutive series of 36 patients with CLM either resected for cure if unresectable, or biopsied. All patients were operated on between 16 September 1988 and 28 July 1992. Liver metastases were histologically confirmed. None of the patients had received chemotherapy or radiation therapy prior to surgery. The site of the primary tumour was the rectum in 17 patients, the left colon in 17 and the right colon in two. The patients comprised 19 men and 17 women with a mean age at the time of diagnosis of 64.5 (SD $\pm$ 9.5) years (range 36–77 years). Pre-operative staging included carcinoembryonic antigen (CEA) serum levels, computed tomography (CT) or magnetic resonance imaging of the abdomen and pelvis, ultrasonography of the liver with biopsy, chest X-ray and a barium enema or colonscopy. Intraoperative ultrasound of the liver was routinely used to detect occult metastases, to define adequate resection line or to assist a segmental orientated surgical approach. The number of involved segments, the proportion of hepatic replacement (less than 25%, 25–50%, more than 50%) and histological grade of liver metastases were evaluated. Twenty-six patients underwent surgery for synchronous metastases and 10 for metachronous lesions. Clinicopathological

Table 1. Clinicopathology of 36 patients with liver colorectal metastases

	No. of patients
Sex	
Male	19
Female	17
Age (years)	
<65	15
≥ 65	21
Number of segments involved	
1–3	20
≥ 4	16
Proportion of liver involved by tumour (%)	
<25	15
25–49	18
≥ 50	3
Liver resectability	
Resectable	13
Non-resectable	23
Histological grade	
G1	7
G2	18
G3	11

data for all patients were available (Table 1). Resectional therapy of liver metastases was performed in 13 patients, while 23 patients were found to be unresectable. Chemotherapy was the main nonsurgical treatment used, administered with intra-arterial and/or intravenous infusions, mainly using 5-fluorouracil. Follow-up programmes included CEA assay, liver biochemistry, liver ultrasonography every 3 months and chest X-ray, CT of abdomen and pelvis every 6 months for 2 years, then yearly.

Tissue handling

Multiple samples of the liver metastases were taken from different representative areas immediately after surgical resection or biopsy. In 16 patients with multiple hepatic lesions, the biopsy sample was taken from at least two representative lesions. All metastatic liver tumours measured more than 5 mm in diameter. All tissues were carefully trimmed to remove as much non-neoplastic tissue as possible, avoiding any nonviable areas. Furthermore, samples of normal hepatic or colon tissue (histologically free from tumour) were taken from a corresponding non tumour area as far as possible from the tumour site, to be used as a standard reference for molecular genetic and flow cytometric analyses. The tissues were bisected and one-half of each sample was processed for conventional histopathological examination and immunohistochemical analysis, while the remaining half was frozen immediately and stored at -80°C until being analysed. The adequacy of the material was checked on frozen tissue sections and only tissue samples with more than 60% tumour content were studied. Evaluation of each biomolecular variable (MIB-1, DNA-ploidy, SPF, *p53* and *Ki-ras* mutations) was carried out independently and without knowledge of the clinical data.

Immunohistochemistry: MIB-1 staining and scoring

Sections (4 μm) from the formaldehyde-fixed, paraffin-embedded aliquots of each specimen were deparaffinized by routine procedures and rehydrated. The sections were then incubated in a microwave oven twice for 5 min each at 750 W in citrate buffer (pH 6.0) and left to cool for 20 min. Immunostaining was performed by means of the ABC (avidin-biotin complex) method. After the addition of normal rabbit serum the sections were first incubated at room temperature with MIB-1 monoclonal antibody (1:50 Immunotech, Marseille, France) for 60 min. Slides were subsequently incubated with a secondary biotinylated rabbit anti-mouse antibody and the avidin-biotin complex (both from Dakopatts, Copenhagen, Denmark), developed with diaminobenzidine (Sigma, St Louis, MO) and counterstained with haematoxylin. Between each step of the procedure, washes were performed with several changes of phosphate-buffered saline (PBS). Sections from a human tonsil were used as positive and negative (omitting the primary antibody) control slides in every staining batch. MIB-1 immunostaining was scored using a standard Leitz Orthoplan microscope (Z 40 objective) with an eyepiece of 10×10 mm^2. All tumour cells which showed a distinct brown staining of the nuclei (Figure 1) were counted as MIB-1-positive. Areas of necrosis were excluded. MIB-1 labelling index (MIB-1 LI) was determined by scoring a total of 1000–3000 tumour cells on 5–10 high-power fields of each tumour, and was calculated as the percentage ratio between immunostained nuclei and total tumour cells. Immunostained sections were analysed by two independent observers (RMT and VM); in the few cases in which the evaluation provided different results, a consensus interpretation was reached after re-examination. The immunostained sections were evaluated blindly, with no knowledge of the clinical or biomolecular data.

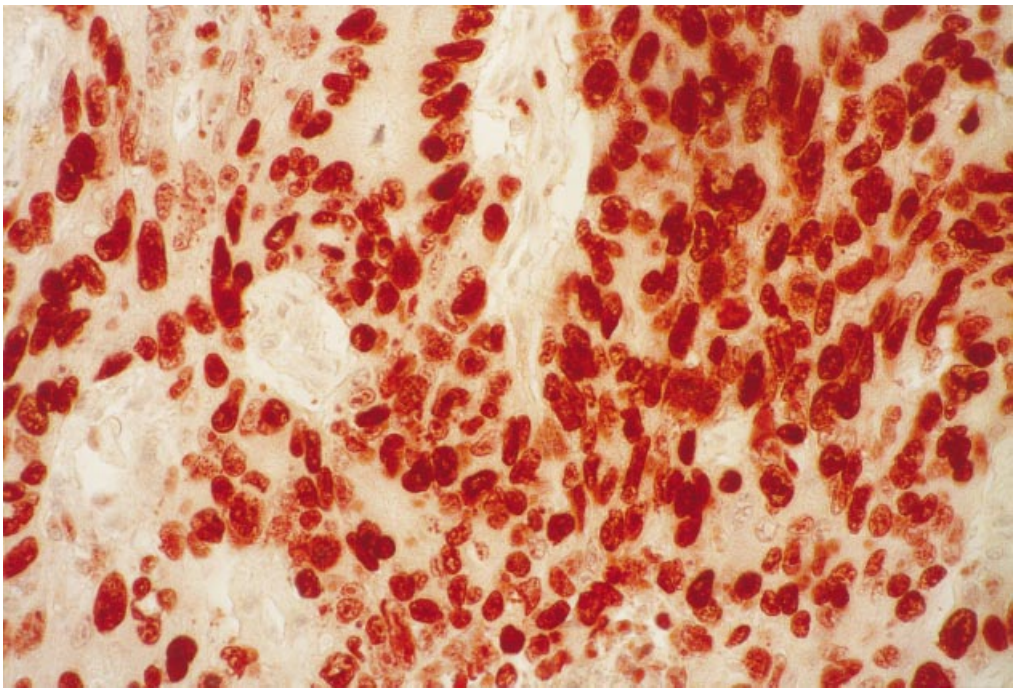

Figure 1. Crowded strongly stained nuclei showing MIB-1 immunoreactivity (ABC immunoperoxidase; original magnification $\times 400$).

Cellular DNA-content and S-phase fraction flow-cytometric examination

DNA flow cytometry was performed on mechanically disaggregated samples of frozen tumour tissue as previously described (Russo *et al.* 1991). A laser flow cytometer (Ortho Diagnostic Systems K.K.; Matusaki Tateisi Electronics Co., Japan) was used for data acquisition. DNA histogram analysis was carried out by means of the Multicycle Software Program (Phoenix Flow Systems, San Diego, CA), including systematic background subtraction (Dean & Jett 1974, Rabinovitch 1990). DNA-ploidy, DNA index and S-phase fraction (SPF) were determined as previously reported (Russo *et al.* 1994).

Detection of *p53* and *Ki-ras* gene mutations

DNA extraction

High molecular weight genomic DNA was extracted as previously described (La Farina *et al.* 1993) from metastatic lesions in the liver and normal liver or colon (as internal control) specimens which had been stored frozen at −80°C and then pulverized in liquid nitrogen by Mikro-Dismembrator U (B. Braun, Melsungen AG, Braun Apparate, 3508 Melsungen, Germany). In one of the 36 patients p53 and *Ki-ras* analysis could not be performed because material was not available.

DNA amplification

Mutations within the *p53* and *Ki-ras* genes were detected by SSCP analysis following PCR amplification of the region of interest. PCR amplification of the first coding exon of the *Ki-ras* gene was performed as described previously (Albanese *et al.* 1997). Exons 5–8 of the *p53* gene were amplified individually from genomic DNA, using four pairs of specific intronic oligonucleotide primers, previously described by Cripps *et al.* (1994). All PCR reactions were carried out in a final volume of 100 μl containing 0.5 μg genomic DNA aliquots and 200 μM dNTPs and 50 pmoles of each amplimer in 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3). After denaturation at 94°C for 5 min, 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Branchburg, NJ) were added to each sample. The samples were subjected to 30 cycles (30 s at 94°C, 30 s at 58°C, 60 s at 72°C) followed by 10 min incubation at 72°C. In every instance, negative (DNA was replaced with water) controls were amplified by polymerase chain reaction (PCR) and included in the experiment. In all PCR assays aerosol-resistant pipette tips were used to avoid cross-contamination. The quality and the concentration of the amplification products were verified by 1.5% agarose gel electrophoresis and ethidium bromide staining.

SSCP analysis

One-hundred nanogram aliquots of the amplified DNA fragments, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA), were denatured (Yap & McGee 1992) and analysed by electrophoresis (*Ki-ras* at 20°C or 22°C; *p53* exon 5 at 22°C, *p53* exon 6 at 4°C, *p53* exon 7 at 10°C and *p53* exon 8 at 13°C) on a 20% polyacrylamide gel ($8 \times 15 \times 0.1$ cm) in TBE (90 mM Tris-borate, 2 mM EDTA) buffer, at 400 V for 1.5–2 h, essentially as described by Hongyo *et al.* (1993). In order to keep the temperature constant, the electrophoretic run was performed in a DGGE-2000 System (C.B.S. Scientific Company, Del Mar, CA) equipped with a KR-50 A immersion chiller (PolyScience, Niles, IL). After the run, the gel was stained for 20 min with 0.5 μg/ml ethidium bromide in TBE and destained for 5 min; the DNA fragments were visualized under UV light. PCR-SSCP analysis was

repeated twice for each sample to minimize the possibility of artefacts due to contamination or polymerase errors. DNA of normal hepatic or colon tissue from each patient was also amplified and run in parallel with matched tumoral DNA samples on SSCP gels, to evaluate the occurrence of germline mutations or polymorphisms.

DNA reamplification and sequencing

Individual ssDNA fragments with shifted mobilities, compared to normal control, were electroeluted from polyacrylamide gel, reamplified and sequenced as described previously (Albanese *et al.* 1997).

Statistical analysis

Because of their asymmetric distribution, the correlation between the proliferative variables (MIB-1 LI and SPF) was evaluated by the nonparametric Spearman correlation. Fisher's exact test (StatXact Turbo, Cytel Software Corporation, Cambridge, MA) was used to test the associations between biological variables. The relationship of different prognostic variables to overall survival was assessed univariately by means of the Kaplan-Meier method (Kaplan & Meier 1958). The prognostic variables tested were sex and age of the patients, number of involved segments, proportion of hepatic replacement (less than 25%, 25–49%, more than 50%), liver resectability, histological grade, DNA-ploidy, SPF, MIB-1 LI, *p53* and *Ki-ras* mutations. Survival time was calculated from the date of surgery to the date of death (cancer-related causes) or last follow-up, with times censored for patients dying of causes unrelated to CLM. Significant differences among survival curves were checked by the log-rank test and Wilcoxon test, or a test for trend when appropriate (Peto *et al.* 1977). Multivariate analysis was carried out by means of Cox's logistic regression model, using a backward procedure (Cox 1972). *P*-values lower than 0.05 were considered significant.

RESULTS

MIB-1 staining

This was predominantly granular and appeared located both at the periphery of the nuclei and in perinuclear regions. In most cases there was a widespread pattern of staining distribution. Some variation from field to field was seen in staining intensity, but, on the whole, positive nuclei were readily identifiable. The range of MIB-1 LI was 8.7–52.6% and the median score was 18.8% (interquartile range: 15.1–23.6%). The cut-off point of 18.8% (median value of MIB-1 LI) was used to discriminate between low and high MIB-1 LI tumours.

Cellular DNA content and S-phase evaluation

Adequate DNA histograms were obtained for all normal and tumoral tissues by means of flow cytometry. The coefficients of variation of the DNA-diploid peak ranged from 2.3% to 5.7% (mean 3.6%). DNA-aneuploidy was found in 78% of the cases (28/36); 7% of these (2/28) showed multiclonality. The SPF ranged from 6.8% to 32.4% (median: 20.3% and interquartile range: 16.6–23.6%). The median SPF of DNA-aneuploid tumours was 20.5% while that of DNA-diploid tumours 19.1% (P=not significant). By using the SPF median value as the cut-off point, tumours were accordingly divided into low ($\leqslant$ 20.3%) and high ($>$ 20.3%) SPF tumours.

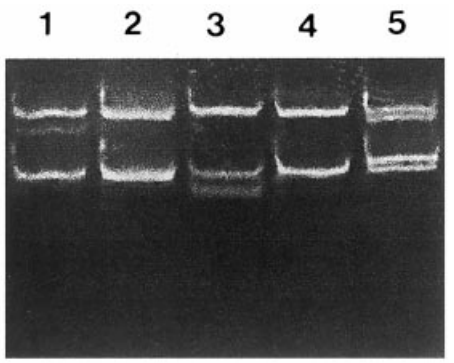

Figure 2. SSCP analysis of exon 6 of the *p53* gene, amplified from colorectal liver metastases genomic DNA of 5 patients. In the samples shown in lane 2 (tumour 41) and lane 4 (tumour 75), only the wild type ssDNA fragments can be detected. The extra bands visualized in lane 1, 3 and 5 correspond to ssDNA molecules harbouring mutations in codon 216 (GTG to ATG, tumour 880), 213 (CGA to CGG, tumour 415) and 220 (TAT to TGT, tumour number 184), as confirmed by sequencing.

Mutation analysis of *p53* and *Ki-ras* genes

Mutation analysis of exons 5–8 of the *p53* gene was performed on genomic DNA from liver metastases of 35 patients by the PCR-SSCP technique. Sequence analysis of the DNA fragments with altered electrophoretic mobility allowed the establishment of the exact site and nature of the genetic alteration. Figure 2 shows a typical SSCP pattern of mutations in exon 6 of the *p53* gene. Aberrantly migrating bands were found in 43% (15/35) of the cases. Three tumours were found to harbour two different *p53* mutations. The distribution of the 18 mutations was: 11% (2/18) in exon 5, 28% (5/18) in exon 6, 50% (9/18) in exon 7 and 11% (2/18) in exon 8. Missense mutations were predominant (15/18, 83%) (Table 2). No germline

Table 2. List of individual liver colorectal metastasis showing *p53* mutations

Tumour	Exon	Codon	Base change	Results
427	5	141	TGC to TAC	Cys to Tyr
538	5	152	1 bp insertion	Frameshift
415	6	213	CGA to CGG*	Arg to Arg
880	6	216	GTG to ATG	Val to Met
184	6	220	TAT to TGT	Tyr to Cys
418	6	220	TAT to TGT	Tyr to Cys
753	6	220	TAT to TGT	Tyr to Cys
1026	7	244	GGC to AGC	Gly to Ser
1149	7	244	GGC to AGC	Gly to Ser
124	7	248	CGG to TGG	Arg to Trp
1150	7	244	GGC to AGC	Gly to Ser
		248	CGG to TGG	Arg to Trp
294	7	244	GGC to AGC	Gly to Ser
		249	1 bp deletion	Frameshift
817	7	244	GGC to AGC	Gly to Ser
		249	1 bp deletion	Frameshift
131	8	273	CGT to CAT	Arg to His
464	8	273	CGT to CAT	Arg to His

*Listed among the mutations since in the matched nontumoral DNA sample only the CGA codon was detected. bp, base pair.

Table 3. Distributions of K-ras gene mutations in 35 liver colorectal metastasis

Mutated codon	Mutated sequence (amino acid)	No. of mutation (%)
12	GAT (Asp)	6 (40)
	GTT (Val)	2 (13)
	TGT (Cys)	1 (7)
13	GAC (Asp)	6 (40)

Wild-type sequences at codon 12 and 13 are GGT (Gly) and GGC (Gly), respectively.

mutations were found, indicating that the changes were somatic. Parallel analyses of exon 1 of *Ki-ras*, in which over 85% of the reported mutations of this oncogene are known to occur, revealed that 15 (43%) of the 35 CLM DNA specimens harboured point mutations. 60% (9/15) of these were in codon 12 (Table 3). Examples of *Ki-ras* mutations detected by SSCP analysis are shown in Figure 3.

Relationship between proliferative activity and genetic alterations

As shown in Figure 4, Spearman analysis showed a high correlation (rho=0.81; $P<0.01$) between the MIB-1 LI and SPF value. Moreover, *p53* gene mutations were associated with high MIB-1 LI and with high SPF (Table 4). There was no relationship between *Ki-ras* gene mutations or DNA-ploidy and the other biological factors.

Uni and multivariate analysis of prognostic factors

The median follow-up time in our study group was 33 months (range 4–66 months). The median survival of the whole group was 16 months. On univariate analysis, variables significantly associated with risk of death were: more than four involved segments, 25% to more than 50% of hepatic replacement, nonresectability, high SPF and high MIB-1 LI (Table 5). The association between overall survival and DNA-ploidy or *p53* mutations did not reach statistical significance, even though a slightly better survival was observed for patients with DNA-diploid tumours or without mutations ($P=0.05$ and $P=0.06$, respectively) (Table 5). Moreover, no differences in survival were observed by stratifying patients according to the site of the *p53* mutations (data not shown). Univariate analysis also failed to reveal any significant associations between *Ki-ras* or *Ki-ras* plus *p53* mutations (data not shown) and

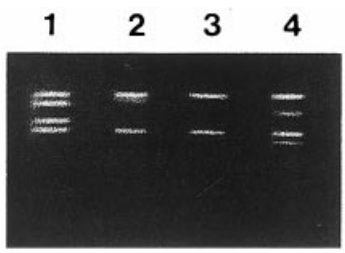

Figure 3. SSCP analysis of exon 1 of *Ki-ras*, amplified from colorectal liver metastases genomic DNA of four patients. In the sample shown in lane 3, only the wild-type ssDNA fragments can be visualized; the extra bands detectable in lane 1, 2 and 4 correspond to ssDNAmolecules containing codon 13 mutated to GAC (lane 1), codon 12 mutated to GTT (lane 2) and codon 12 mutated to TGT (lane 4), as confirmed by sequencing.

patient survival. However, when the impact of *Ki-ras* mutations in individual codons was evaluated, the overall survival was slightly shorter in patients with codon 13 mutations compared with those with mutations in codon 12 or no mutation, though the difference did not reach statistical significance (Table 5). The significant variables at the univariate level were entered into a multivariate logistic regression model with backward elimination. Only two variables remained statistically significant as independent predictors in the final model. These were liver resectability (hazard ratio, 5.30; $P<0.01$) and SPF (hazard ratio, 3.13; $P<0.01$) (Table 6). Figure 5 shows the probability of overall survival in relation to SPF.

DISCUSSION

Basic uncertainty still exists regarding the prognostic value of classical indicators which might have strong impact on the outcome of CLM. It is therefore extremely important to define

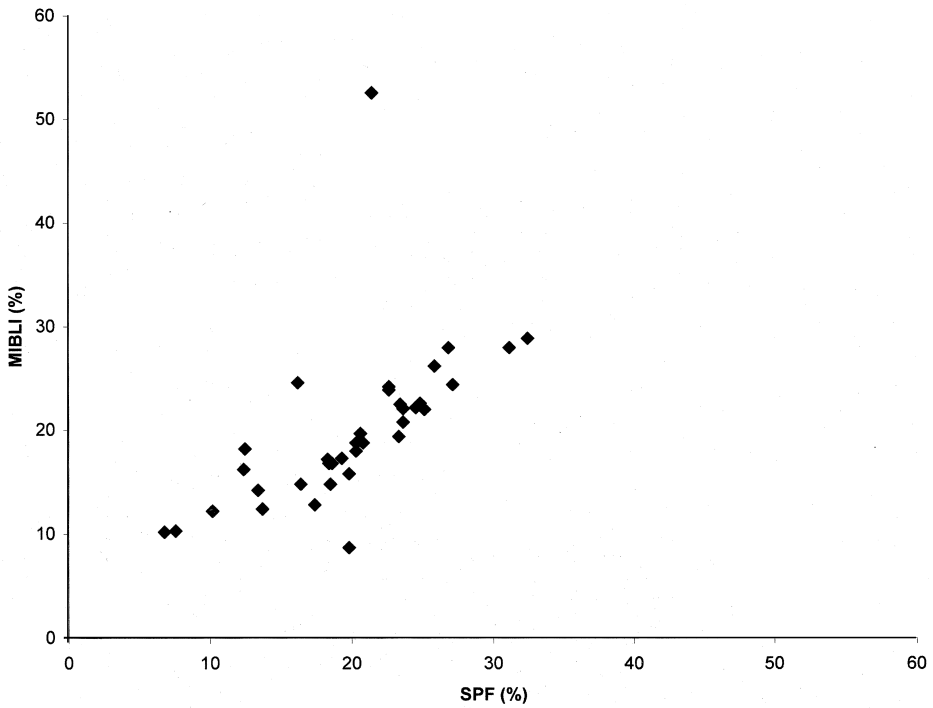

Figure 4. Correlation between MIB-1 LI and flow cytometric S-phase fraction (SPF) (rho = 0.81; $P < 0.01$; $n = 36$).

Table 4. Relationships of p53 to S-phase fraction (SPF) and MIB-1/LI

	No. of points	SPF (median)	*P*-value	MIB-1/LI (median)	*P*-value
p53					
Wild-type	20	18.4		16.8	
Mutated *p53*	15	23.4	<0.01	22.2	<0.05

Table 5. Univariate analysis of overall survival (OSV): distribution of patients for all variables examined according to Kaplan–Meier method

	No. of patients	% OSV		O/E	*P*-value
		12 months	24 months		
Total	36	69	33		
Number of segments involved					
1–3	20	90	50	0.71	
≥4	16	44	12	1.79	<0.01
Proportion of liver involved by tumour (%)					
<25	15	87	60	0.59	
25 to ≥50	21	57	14	1.67	<0.01
Liver resectability					
Resectable	13	100	77	0.49	
Non-resectable	23	52	9	1.83	<0.01
DNA-ploidy					
Diploid	8	62	50	0.85	
Aneuploid monoclonal	26	77	31	0.99	0.05
Aneuploid multiclonal	2	0	0	4.61	
SPF					
≤20.3	19	84	57	0.69	
>20.3	17	53	6	1.90	<0.01
MIB-1/LI					
≤18.8	18	82	52	0.72	
>18.8	18	58	12	1.73	<0.01
p53					
Wild-type	20	71	47	0.80	0.06
Mutated *p53*	15	67	13	1.52	
Ki-ras					
Wild-type	20	60	29	1.02	
Mutated at codon 12	9	89	56	0.78	ns
Mutated at codon 13	6	67	17	1.50	

O/E, observed/expected.

Table 6. Multivariate regression analysis to predict the RR of death in patients with liver metastasis

	Hazard ratio (95% CL)	Beta	*P*-value
Liver resectability (Non-resectable vs. resectable)	5.30 (2.10–13.4)	1.669	<0.001
SPF (>20.3 vs. ⩽20.3)	3.13 (1.35–7.28)	1.141	<0.01

CL, confidence limit.

new biological factors that may help to recognize the more aggressive types of such neoplasias. Identification of low and high-risk groups is of major importance in the choice of significant criteria for planning adjuvant therapy or alternative treatments and follow-up of these patients.

Proliferative activity of neoplastic cell populations appears to be a major determinant of biological aggressiveness. Its evaluation provides useful information in identifying patient subgroups with higher risk (Silvestrini 1994, Tomasino *et al.* 1995). It is therefore crucial to use an accurate, reproducible and easy-to-use technique for this type of analysis. In the present prospective study we compared SPF and MIB-1 LI values, which represent two different theoretical and methodological approaches to measure cell kinetics. SPF essentially indicates the proportion of cells synthesizing DNA, although (as discussed below) its determination might be affected by several factors (Meyer & Coplin 1988). MIB-1 (a true equivalent of Ki-67 (Key *et al.* 1992)) immunoreactivity has been used for assaying the growth fraction by immunohistochemistry, because the corresponding antigen, detected in proliferating cells at any phase of the cycle (Gerdes *et al.* 1984), is not expressed in G_0 (Lopez *et al.* 1991, Schluter *et al.* 1993). If Ki-67 were indeed expressed in all proliferating cells, at all phases of the cycle, then the absolute values of MIB-1 should be expected to be higher than those of SPF. However, a significant correlation between MIB-1 and SPF has

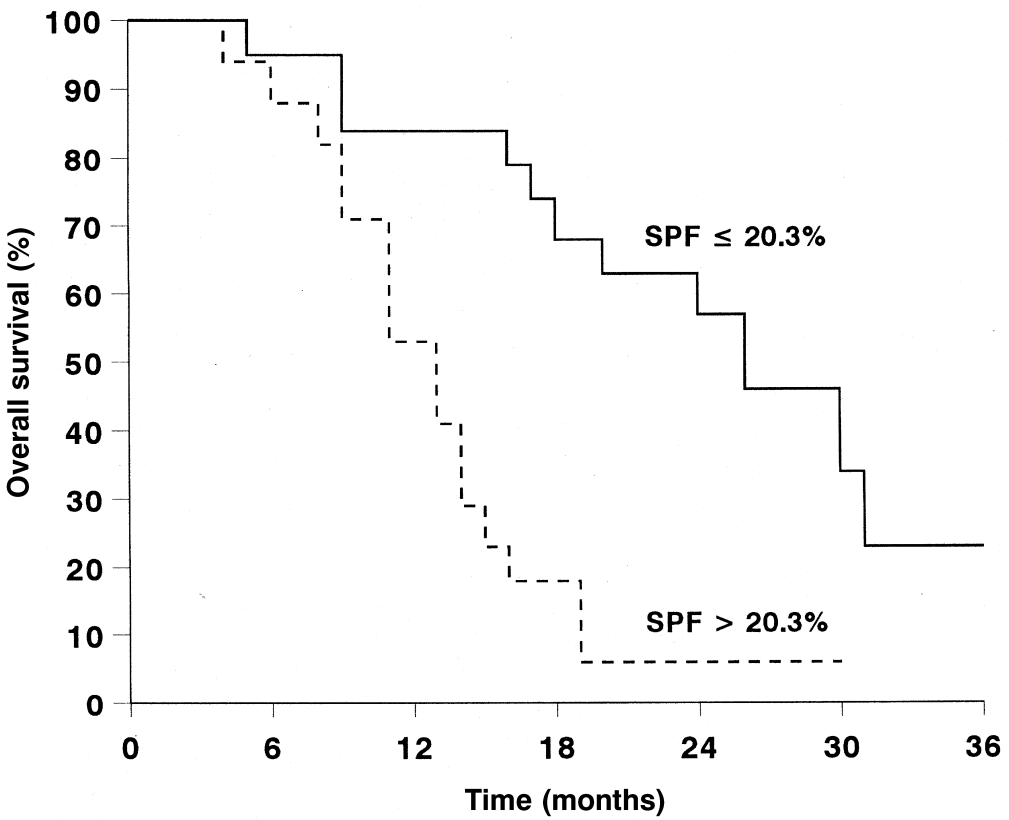

Figure 5. Probability of overall survival according to flow cytometric S-phase fraction (SPF) ($\leqslant$ 20.3% vs. $>$ 20.3%, $P < 0.01$) in CLM patients.

been reported previously for some human tumours (Pich *et al.* 1994, Ellis *et al.* 1996). To the best of our knowledge, there have been no previous studies in CLM. Our present data show a high correlation between these two proliferative indices also in these tumours. Indeed, in our series the median value of SPF was even slightly higher than that of MIB-1 LI. This finding agrees with that of Ellis *et al.* (1996) in a similar study performed on human breast carcinoma. An overestimate of the fraction of cells in S phase by flow cytometric determination can not be ruled out, due to the presence of debris, an admixture of nonepithelial cells or S-phase arrested cells (Meyer & Coplin 1988), and also because, as suggested by Ellis *et al.* (1996), in tumours with very low proliferative activity any error in the estimate of the percentage of cells in S-phase could be emphasized. However, this alone can not justify the significant correlation observed in our and other studies between SPF and MIB-1. Rather, while adding further support to previous evidence that Ki-67 is selectively expressed in proliferating cells, this finding suggests that, at least in the tumour samples analysed, it is expressed, or at least detectable, only in a portion of the cell cycle, similar in duration to the S-phase. Indeed, as shown in HL-60 and MCF-7 cells the Ki-67 level of expression is very low in G_1 and early S-phase, rises rapidly in late S, reaching a peak in mitosis (Bruno & Darzynkiewicz 1992, Santisteban & Brugal 1994). In addition, some tumour cells arrested in S-phase might not express any Ki-67 antigen, and thus not be recognized by MIB-1, as previously shown by Van Dierendonck *et al.* (1989) and Vielh *et al.* (1990) for Ki-67. Thus, an unambiguous interpretation of the immunohistochemistry data in terms of proliferative activity of a cell population must wait until the physiological role of this protein, and a better characterization of its level of expression through the cycle in different cell types, becomes available. In any event, from our data, MIB-1 LI, although associated with an adverse outcome in univariate analysis, does not appear to be of a major prognostic importance, not behaving as an independent prognostic factor in multivariate analysis, when compared with the other variables. Nevertheless, longer observation periods and larger sample sizes are needed before a conclusion can be reached regarding this point. In contrast, our study showed that SPF is a strong prognostic indicator for overall survival in patients with CLM. This finding also shows that SPF maintains its predictive role even in the presence of other well-established independent prognostic variables for CLM, such as number of involved segments, the proportion of hepatic replacement and liver resectability. The traditional parameters selected for the analysis are known to play an independent role in the prognosis of patients with CLM (Tranberg & Bengmark 1994). Our report confirms and reinforces a previous prospective study on the prognostic role of cell kinetics, evaluated as [3H]thymidine labelling index, on both freedom from progression and overall survival in CLM (Silvestrini *et al.* 1990). Measurement of cell proliferation might provide prognostic information and a rational basis to develop more specific clinical treatments for the group of patients with resectable CLM. Moreover, it might allow a prediction of the response to cell cycle dependent therapies such as chemotherapy for the group of patients with unresectable CLM. However, how the evaluation of the proliferative activity should be inserted in the choice of therapeutic management of CLM remains to be established.

The frequency of DNA-aneuploid tumours observed in our study (78%) was among the highest published values (Kokal *et al.* 1986, Tsushima *et al.* 1987, Yamaguchi *et al.* 1990, Cady *et al.* 1992, Lind *et al.* 1992). This is probably due to the use of multiple tissue sampling of all cases studied, which greatly reduces the probability of missing DNA-aneuploid clones, and to the sample-preservation method chosen (freezing at -80°C), which permits a higher histogram resolution. Previous studies on the prognostic significance of DNA-ploidy status in CLM have provided conflicting results. Several authors proposed DNA-ploidy as a significant

prognostic indicator in CLM (Tsushima *et al.* 1987, Yamaguchi *et al.* 1990, Cady *et al.* 1992), even though, the independent prognostic value of DNA-ploidy was assessed by multivariate analysis only in two of these series (Yamaguchi *et al.* 1990, Cady *et al.* 1992). In contrast, Kokal *et al.* (1986) reported a series of 53 CLM in which DNA-ploidy was not associated with a shorter survival. Similarly, Lind *et al.* (1992), who evaluated 37 patients with CLM, failed to find any relationship between DNA content and survival. Our data also support the conclusion that overall survival and DNA content of the CLM are not associated. Despite the small number of cases investigated in our study, our findings suggest that once liver metastases are present, DNA-ploidy may have no effect on survival. Thus, while as shown by our own (Russo *et al.* 1991) and other studies, DNA-ploidy status is a prognostic marker in localized colorectal carcinomas (Bauer *et al.* 1993), it loses its relevance in more advanced stages (Kokal *et al.* 1986, Lind *et al.* 1992).

The frequency of *p53* mutation detected by SSCP analysis and sequencing in our series of CLM was 43% (15/35), lower than that observed in other studies, in which fewer cases of CLM were examined (Kastrinakis *et al.* 1995, Yao *et al.* 1996). The mutation frequency of *p53* observed in liver metastases was higher than that in paired primary colorectal carcinomas (unpublished data). A similar finding has been reported by others (Kastrinakis *et al.* 1995, Yao *et al.* 1996) and interpreted as evidence for a possible role of *p53* mutation in conferring metastatic ability to cancer cells. In our study, we found an association between *p53* gene mutation and proliferation activity as determined by MIB-1 or SPF. This finding, obtained in our *in vivo* analysis, confirms the idea that point mutations of *p53* effectively remove the regulatory influence of wild-type p53 protein on the cell cycle and may lead to an uncontrolled or accelerated cellular proliferation. Moreover, in the present report, the overall survival time was shorter in patients with *p53* mutations, although it was not significantly different. In contrast, in primary colorectal carcinomas these mutations were found to be clearly associated with a poor prognosis (Hamelin *et al.* 1994, Goh & Smith 1995, Pricolo *et al.* 1996).

No significant difference in survival was found between the *Ki-ras* wild-type and mutant groups from the survival analysis of our series. An interesting finding was a slightly worse prognosis for tumours with mutation in codon 13, although statistical significance was not reached, probably because of the small number of cases showing this mutation. This latter result is in accordance with our previous report (Albanese *et al.* 1997) on primary colorectal carcinomas where we observed that mutations in different codons of *Ki-ras* appear related to different biological features. In particular, *Ki-ras* mutations in codon 13 were found to be associated with Dukes' D stage in primary carcinomas and thus possibly related to a more aggressive phenotype.

In conclusion, the results from this study suggest that SPF, more than MIB-1 LI, may be a useful prognostic factor for better stratification and for a more efficient therapeutic choice in patients with colorectal liver metastases. Moreover, although genetic alterations did not reach a prognostic significance in CLM, perhaps because of the limited patient cohort, nevertheless the finding of a significant association between high SPF and *p53* mutations provides a hint to the biological cause of the greater proliferative activity of some tumours, responsible in turn for their highly aggressive phenotype.

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