Determination of Molecular Marker Expression Can Predict Clinical Outcome in Colon Carcinomas

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ABSTRACT

Purpose: **Conventional staging procedures are often unable to precisely predict prognosis in colorectal cancer (CRC). In this study, we set out to investigate the possible role of molecular/structural indicators involved in cell cycle regulation (p27 and p53), apoptosis (p53 and p27), and tumor neoangiogenesis [p53, vascular endothelial growth factor (VEGF), and microvessel count] in predicting tumor behavior and clinical outcome in CRC patients**

Experimental Design: **Analysis of the above indicators was performed by immunohistochemistry on 104 CRC patient samples and 25 normal colon mucosa specimens.**

Results: **Intense p27 nuclear staining was found in normal colon mucosa, with p53 nuclear staining and VEGF cytoplasmic accumulation <10%, and low microvessel count. In contrast, in CRC samples, p27 was down-regulated in 53.8%, p53 protein was overexpressed in 52%, and VEGF stained positive in 67.3% of the cases, respectively. Multiple regression analysis showed that molecular markers were strongly correlated. In patients treated with curative surgery, a significant relationship was seen between p27 down-regulation and Dukes' stage, nodal status, and the presence of distant metastases. VEGF overexpression correlated significantly with Dukes' stage, tumor (*t*) and metastasis (*m*) parameters, and left site. Stepwise regression selected p27, p53, VEGF, and Dukes' stage as the best combination of variables capable of predicting both disease-specific and disease-free survival.**

Conclusions: **The investigated indicators may be useful for the prediction of outcome and recurrence rate in curatively treated CRC patients. In conjunction with clinical and pathological staging, they may provide a stronger indication of clinical outcome than staging alone and help better select therapeutic options in CRC patients.**

INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of cancer death worldwide, accounting for more than 150,000 new cases and 55,000 deaths in the United States every year and ~125,000 mortalities each year in Europe (1, 2). To date, radical surgery, followed by adjuvant chemotherapy when appropriate, is the mainstay of therapy for patients with localized disease (3–6). However, despite adjuvant therapies, a significant proportion of patients presents with recurrence; moreover, patients with the same tumor stages may show different outcomes, indicating that the conventional staging procedures may be unable to precisely predict cancer prognosis (7, 8). Therefore, the search for new prognostic factors capable of identifying high-risk patients and of modulating cancer treatment options is still actively ongoing (9). In this regard, many studies have focused on innovative molecular markers playing an important role in cell cycle regulation, apoptosis, and tumor neoangiogenesis.

Uncontrolled cell proliferation is the hallmark of cancer, and there is increasing evidence that tumor cells have a damaged cell cycle-regulatory machinery. p27, an inhibitor of cyclin-dependent kinases that regulates the G_1-S phase by blocking the cell cycle and maintaining cells in resting state (G_0), seems to plays an important role in the negative regulation of cell growth (10–12). Low or absent p27 protein expression has been described in the cells of a large variety of human tumors, including CRCs (3, 13). However, contrasting results have been reported on the association between low or absent p27 protein and both survival and recurrence rate (12, 14). Thus, the consequence of low or high p27 protein expression in CRCs still remains unclear.

The *p53* tumor suppressor gene has various important functions in cellular integration, including response to DNA damage, regulation of transcription, and control of genomic stability. In addition to cyclins, p53 influences the G_1-S-phase checkpoint, and plays a pivotal role in apoptosis (15). The loss of the cell cycle apoptotic control mechanism through p53 mutation is believed to be one of the most important mechanisms of tumorigenesis in many human tumors (16). Mutations in the *p53* gene with overexpression of its protein product are present in up to 70% of CRCs and have been proposed to be a late event in the progression from adenoma to colon carcinoma, after APC and K-ras mutations (17). A correlation between p53 alteration and poor prognosis has been demonstrated in many studies (3, 18); however, some investigations have reported

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none, or even an improved, clinical outcome for colon tumors overexpressing p53, and a recent meta-analysis showed discordant results between p53 and prognosis in CRCs (3, 19). Thus, the prognostic significance of p53 alterations in CRCs remains controversial and needs additional studies to be clarified.

The *p53* tumor suppressor gene is also involved in tumor angiogenesis, and a deregulation of p53 protein function has been associated with increased neovascularization and aggressive tumor growth (20). Tumor growth requires neoangiogenesis, and angiogenesis is known to initiate imbalance between cell proliferation and apoptosis (21). Several positive regulators of tumor angiogenesis have been identified. Among these, vascular endothelial growth factor (VEGF) seems to play a crucial role in the proliferation and migration of endothelial cells, providing nourishment to the growing tumor and making the tumor cell establish continuity with the host vasculature (4). It has been suggested that neoangiogenesis can be assessed using microvessel count (MVC) determined immunohistochemically with a monoclonal antibody against the endothelial surface marker CD34 (22). Significant correlations between VEGF overexpression and high MVC, and between p53 protein accumulation and high VEGF expression have been reported in CRCs; moreover, some studies have demonstrated a more aggressive behavior in colon cancers overexpressing VEGF and/or showing a high frequency of microvessels (23). However, other investigations have been unable to show any correlation between levels of neoangiogenetic markers and molecular patterns or clinical outcome, thus warranting additional analyses (24, 25).

In the quest for novel prognostic factors, we have already subjected cytokine serum levels, DNA ploidy, and genetic alterations in CRCs to prognostic assessment (9, 26, 27) over the last few years. In this study, the above mentioned conflicting results prompted us to investigate the possible correlations of p27, p53, VEGF, and MVC with each other, with well-known prognostic indicators, and, finally, with clinical outcome, in an effort to identify high-risk colon cancer patients to whom to administer tailored cancer treatment options.

PATIENTS AND METHODS

Patients. The study population consisted of 104 consecutive patients (73 male, 31 female; age range, 28–84 years; mean 63.8 ± 11.9 years) observed between January 1995 and December 2000, with histologically proven colon carcinoma. Patients meeting the Amsterdam II criteria for hereditary nonpolyposis CRC syndrome or with carcinomas associated with inflammatory bowel disease or with rectal cancers were not considered suitable for this study because their molecular features, recurrence rate, and overall survival can differ greatly from sporadic large bowel carcinomas, thus possibly causing misinterpretation of the results (15, 28). Twenty-five subjects without colon carcinoma undergoing endoscopic biopsy of normal-appearing colon mucosa served as controls. All of the patients gave their informed consent and the study was approved by the ethical committee of the Department of Clinical and Experimental Medicine and Surgery of the Second University of Naples. All of the tumors were staged on evaluation of the findings of physical examination, routine laboratory tests, and diagnostic imaging (chest X-ray, abdominal ultrasound, chest and abdominal computed tomography scan, scintigraphic bone scan, and endoscopy). All of the patients underwent surgical exploration with intraoperative hepatic ultrasound and tumor removal; no explorative laparotomy or colon by-pass was performed in these patients. Eighty-six patients underwent a potentially curative surgery, defined as the removal of all of the macroscopic tumoral tissue, absence of microscopic residual tumor, free resection margins, and lymphoadenectomy extended beyond involved nodes at postoperative pathological examination. In the remaining 18 patients, the surgery was judged as noncurative or palliative because the primary tumor was not completely removed, or peritoneal or hepatic metastases were present, or metastasis was detected in the more distant resected nodes. No major complications were observed, and all of the patients could be discharged from the hospital. After curative surgery, if case recurrence was suspected, patients were subjected to additional diagnostic methods always complemented by routine histopathological examination of a biopsy specimen. No patient was lost to follow-up, and it was complete by December 31, 2002.

The following parameters were recorded in all patients: age, sex, cancer site (right and left colon by using middle transverse colon as partition), performance status according to the Eastern Cooperative Oncology Group scale, basal carcinoembryonic antigen serum level, type of surgical resection (curative, noncurative), tumor-node-metastasis (TNM) classification and Dukes' stage, degree of histological differentiation (well, moderate, or poor), tumor growth pattern (expanding or infiltrating; Ref. 29), DNA microsatellite stability status (stable and unstable, as assessed by analysis of BAT26 mononucleotide repeat as previously reported; Ref. 27), number of resected nodes, number of metastatic nodes, tumor size, postoperative complications, and recurrences after potentially curative resections.

Immunohistochemical Procedures. Consecutive 4-μm sections were cut from the paraffin blocks of 104 colon carcinoma and 25 normal colon mucosa specimens. Sections underwent histological evaluation to individuate blocks without necrotic and hemorrhagic areas and, for colon carcinoma, histomorphologically representative of the core and the invasive edge of the tumor. Four-μm tissue sections were cut from these defined blocks, placed on charged poly-L-lysine-coated slides, and used for immunohistochemical procedure. The antibodies used were a mouse monoclonal antibody against human $p27^{kip1}$ (clone SX53G8, isotype IgG1; DakoCytomation Norden A/S, Glostrup, Denmark), p53 (clone DO-7, isotype IgG2b; Dako), VEGF (clone JH121, Ab-3; Lab Vision Corporation, Fremont, CA), and CD34 class II (clone QBEnd 10, isotype IgG1; Dako).

The immunohistochemistry procedure was carried out by an automatic immunostainer (Ventana FBMK 750600, Ventana Inc., Tucson, AZ) performing the following automated protocol for BMK 3,3-diaminobenzidine (DAB; 0.7 mg/ml): deparaffination; blockage of endogenous peroxidase activity by incubation with peroxidase blocking reagent kit for 10 min; two-time-antigen-retrieval procedure by microwaving in 10 mmol/L citrate buffer (pH 6.0) for 5 min at 100°C; immunostaining with antibody, 1:100 dilution, in PBS for 32 min at 40°C; incubation with streptavidin-biotin peroxidase complex for 30 min; appli-

cation of chromogen DAB for 5 min; nuclear counterstaining with Mayer's hematoxylin. The slides were dehydrated in graded alcohol solutions, fixed in xylene, and finally mounted on glass coverslips with Entellan (Merck, Darmstadt, Germany). In each analysis, positive controls consisted of colon carcinoma samples known to positively stain for the antibody used, whereas Tris-buffered saline in place of the primary antibody was used as negative control.

Interpretation of Immunohistochemical Staining. Slides were examined by two independent pathologists (F. F., B. A.) blinded to each other's work and with no prior knowledge of clinical and pathological parameters. For each colon carcinoma, staining was evaluated on separate slides in two compartments, at the core and at the invasive edge of the tumor, respectively. Slides were examined at ×400 (40× objective, and 10× ocular), and analysis was performed by counting all of the cells present in the slides avoiding randomization. For each case, at least 5000 cells were observed. All of the slides were independently reviewed twice by each pathologist; discrepancies between investigators (<10% of the cases) required a third joint observation with conclusive agreement. Staining score was expressed as the percentage ratio of stained cells with the total number of cells evaluated (stained cells:total number evaluated). For the purpose of the study and on the basis of previous experience, interpretation of staining score for p53 and VEGF was defined as positive when >10% of tumor cells stained, and negative when none or ≤10% of tumor cells stained, respectively (3, 10, 15, 18, 23, 30). Because the immunoreactivity cutoff for p27 has been reported with a very wide range, from 5 to 50% (2, 10, 12–14), it was decided to use the mean value of p27 nuclear staining to unequivocally categorize cases in two groups: p27 positive (>20% of tumor cells stained) and p27 negative (none or ≤20% of tumor cells stained), respectively. This is in accordance with other recent observations (15). Microvessels were

Table 1 Clinicopathological characteristics

		Resection		
	All (n = 104)	Curative (n = 86)	Noncurative (n = 18)	P^a
Site (right/left)	26/78	22/64	4/14	0.765[b]
Performance status				
0	37	31	6	0.341[b]
1	50	43	7	
2	17	12	5	
Serum CEA[c] levels (ng/ml)[d]	16.2 + 41.5 (0.2–315)	8.9 + 19.7 (0.2–132)	56.2 + 87.2 (1.8–315)	0.075[e]
Tumor classification				
T_1	6	6	0	0.001[b]
T_2	18	18	0	
T_3	75	61	14	
T_4	5	1	4	
Nodes classification				
N_0	71	65	6	0.002[b]
N_1	26	17	9	
N_2	7	4	3	
Distant metastasis, no/yes	84/20	83/3	1/17	0.001[b]
Dukes' stage				
A	21	21	0	0.001[b]
B	44	44	0	
C	19	18	1	
D	20	3	17	
Histological differentiation, well/moderate/poor	6/93/5	6/76/4	0/17/1	0.511[b]
Tumor growth pattern, expanding/infiltrating	58/46	51/35	7/11	0.113[b]
Microsatellite stability status, MSS/MSI	89/15	73/13	16/2	0.660[b]
Resected nodes[d]	12 ± 3 (5–23)	14 ± 2.1 (8–24)	8 ± 1.8 (5–12)	0.001[e]
Metastatic nodes[d]	0.8 ± 1.6 (0–8)	0.6 ± 1.5 (0–8)	1.6 ± 1.7 (0–6)	0.014[e]
Size (cm)[d]	4.7 + 2 (1–13)	4.7 + 2.1 (1–13)	4.9 + 1.6 (3–9)	0.704[e]
P.O. complications, no/yes	92/12	75/11	17/1	0.640[b]
Recurrence after curative resection, no/yes		64/22		

[a] P values refer to curative *versus* noncurative resections.
[b] χ^2 test.
[c] CEA, carcinoembryonic antigen; MSS, microsatellite stability; MSI, microsatellite instability; P.O., postoperative.
[d] Values are mean ± SD (range).
[e] Student's t test.

Table 2 Immunohistochemical staining results

Level	Resection				Multiple regression			
	All (n = 104)	Curative (n = 86)	Noncurative (n = 18)	P^a	p27	p53	VEGF[b]	MVC
p27								
≤20%	56	38	18	0.001[b]		$r = -0.344$	$r = -0.342$	$r = -0.229$
>20%	48	48	0			$P = 0.001$	$P = 0.001$	$P = 0.020$
p53								
≤10%	50	45	5	0.041[b]	$r = -0.344$		$r = 0.314$	$r = 0.206$
>10%	54	41	13		$P = 0.001$		$P = 0.001$	$P = 0.036$
VEGF								
≤10%	34	32	2	0.032[b]	$r = -0.342$	$r = 0.314$		$r = 0.472$
>10%	70	54	16		$P = 0.001$	$P = 0.001$		$P = 0.001$
MVC								
Range	20–235	20–235	29–165	0.956[d]				
Mean (SD)	83 (40)	83 (41)	84 (37)					
95% CI	76–92	74–93	66–103					
Median	73	70	86.5					

[a] P refers to curative *versus* noncurative resections.
[b] VEGF, vascular endothelial growth factor; MVC, microvessel count; CI, confidence interval.
[c] χ^2 test.
[d] Student's t test.

visualized by immunostaining with CD34 and were counted in five regions with the highest vascular density at ×160 (16× objective and 10× ocular); at least 1.685 mm^2 were analyzed. A single microvessel was defined as any brown-immunostained endothelial cell that was separated from adjacent microvessels, tumor cells, and connective tissue elements; large vessels with thick, muscular walls were excluded from the counts. For each sample, the total number of microvessels was determined by adding counts in all five regions of interest. If necessary, the median value was used as a cutoff to divide patients into two groups with high and low MVC, respectively (22).

Statistical Analysis. Statistical analysis was carried out using the BMDP statistical package (BMDP Statistical Software Inc., Los Angeles, CA). In all analyses, the significance level was specified as $P < 0.05$. The equality of group means and comparisons between proportions were analyzed by using unpaired Student's t test and χ^2 test, respectively. Stepwise multiple regression analyzed correlations of different molecular markers with each other, clinicopathological features, and curative and noncurative surgery. The patients who died for causes other than colon cancer without evidence of disease were regarded as censored events for cancer-related mortality rate or disease-specific survival (DSS). The analyses that were related to DSS and disease-free survival (DFS) were restricted to the 86 patients undergoing curative surgery. Univariate statistical analysis was determined by log-rank test (Mantel-Cox) and, for continuous variables, was performed by grouping the patients using the median values as cutoff. The curves were plotted using the product-limit method (Kaplan-Meier) and were analyzed using the Generalized Savage test or Mantel-Cox test (BMDP1L). The independent significance of prognostic variables related to DSS and DFS was determined by multivariate analysis, using Cox's proportional hazards model. The level of significance was obtained by score test (BMDP2L). A stepwise multivariate analysis was performed to generate a model of the best linear combination of variables able to predict DSS and DFS. For covariates retained in the model, relative hazards with 95% confidence interval were estimated. Finally, a Cox model stratified by the different molecular patterns was then constructed according to Kalbfleisch and Prentice (31) to plot cumulative hazard functions for each category. The same model was used to estimate the 5-year probability of death for different combinations of the major covariates. The stratified model allowed good estimates to be made, even in the presence of violations of the proportionality assumption for the variables being used to stratify.

RESULTS

The clinicopathological characteristics of the 104 patients are summarized in Table 1. The tumor-node-metastasis classification system, Dukes' stage, number of resected nodes, and number of metastatic nodes were statistically different between curative and noncurative resections.

Intense nuclear staining for p27 protein (mean, 74 ± 11%; 95% confidence interval, 4%, median, 70%; range from 60 to 90% of the cells observed) was found in all tissue sections of normal-appearing mucosal colon cells from 25 healthy subjects. None of these samples displayed nuclear staining for p53 or VEGF cytoplasmic accumulation greater than 10%. MVC ranged from 31 to 54 (mean, 41 ± 8; 95% confidence interval, 3; median, 41) and was significantly lower than in colon tumors ($P < 0.001$, see Table 2). The immunohistochemical staining results in the 104 colon cancer patients are shown in Table 2. No considerable immunoreactivity differences were noted between the core and the invasive edge of the tumors. All but four colon cancer samples showed nuclear staining for p27 lower than 60% (the lowest value found in nontumoral mucosal cells): mean, 20 ± 17%; 95% confidence interval, 3%; median, 19%; range, 1–80%. According to cutoff values, p27 protein expression was down-regulated in 53.8% of the cases, p53 protein was overexpressed in 52%, and VEGF stained positive in 67.3%. All except

Table 3 Univariate and multivariate analysis related to disease-specific survival in 86 colon cancer patients undergoing curative surgery

	Univariate		Multivariate[a]	
	Hazard rate	*P*	Hazard rate	*P*[b]
Age[c] (>66 yr)	1.238	0.664		
Sex (M/F)	1.119	0.823		
Site (left/right)	1.320	0.628		
PS[d] (>1)	2.596	0.107		
CEA[c] (>2.8 ng/ml)	1.325	0.597		
Tumor (>T_2)	1.793	0.343		
Nodes (>N_0)	3.142	0.009	0.132	0.218
Distant metastasis (Yes)	9.611	0.001	3.887	0.083
Dukes' stage (>B)	3.308	0.001	4.542	0.042
Grading (>2)	1.964	0.232		
Growth pattern (infiltrating)	1.476	0.507		
Microsatellite stability	2.996	0.044	0.128	0.211
Resected nodes (<12)	4.882	0.005	2.487	0.199
No. of metastatic nodes (>2)	3.476	0.019	3.572	0.188
Size[c] (>4.7 cm)	1.531	0.371		
P.O. complications (yes)	1.020	0.981		
p27 (≤20%)	26.555	0.001	9.352	0.004
p53 (>10%)	11.315	0.001	6.012	0.037
VEGF (positive)	11.142	0.003	4.276	0.044
MVC[c] (>70)	4.097	0.007	1.517	0.531

Stepwise multivariate analysis

	χ^2	Hazard rate	95% CI[e]	*P*
Dukes' stage	3.915	1.994	1.44–2.54	0.041
VEGF	5.175	3.623	2.37–4.87	0.023
p53	7.567	5.004	3.50–6.50	0.006
p27	24.855	11.167	9.10–13.22	0.001

[a] Multivariate analysis was performed including variables with significant value on univariate analysis.
[b] Score test (Cox's proportional hazard model).
[c] The median value was used as cutoff.
[d] PS, performance status; CEA, carcinoembryonic antigen; P.O., postoperative; VEGF, vascular endothelial growth factor; MVC, microvessel count.
[e] 95% confidence interval (CI) of hazard rate of cancer-related death associated with higher Dukes' stage, VEGF immunoreactivity, p53 protein overexpression, and low p27 protein accumulation.

CD34 showed a significant difference between curatively and noncuratively treated colon cancers; moreover, multiple regression analysis showed that molecular markers had a strong correlation with each other. No significant correlation was found among molecular markers and age, sex, performance status, basal carcinoembryonic antigen serum levels, tumor grade, tumor growth pattern, microsatellite stability status, number of metastatic nodes, tumor size, and postoperative complications. A significant relationship was seen between p27 down-regulation and Dukes' stage ($r = -0.392$, $P = 0.001$), and between p27 down-regulation and nodal status and the presence of distant metastases ($r = -0.258$, $P = 0.008$; and $r = -0.452$, $P = 0.001$, respectively). VEGF overexpression correlated significantly with Dukes' stage ($r = 0.293$, $P = 0.003$), with tumor (t) and metastasis (m) parameters ($r = 0.256$, $P = 0.001$, and $r = 0.236$, $P = 0.016$, respectively), and with left site ($r = 0.213$, $P = 0.030$). p53 and MVC did not show any correlation with other clinicopathological features; a slight trend was observed between positive p53 protein accumulation and the presence of distant metastases ($r = 0.177$, $P = 0.073$). To summarize, with regard to molecular markers, the lower the level of p27 and the higher the expression of VEGF, the more likely the association with tumor progression, presence of distant metastases, and distal localization.

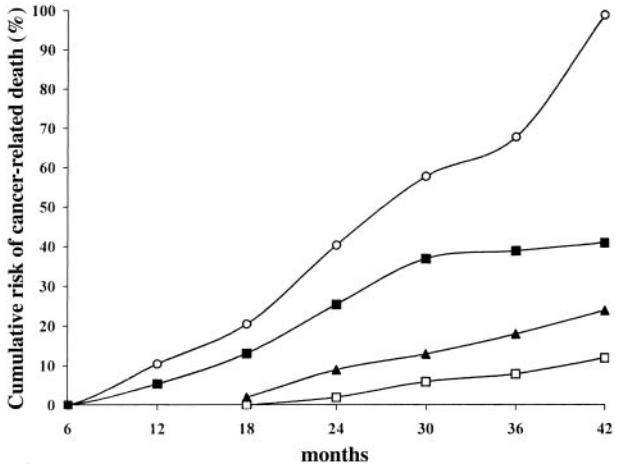

Fig. 1 Multivariate cumulative hazard of cancer-related death function according to different combinations of three molecular markers (p27, p53, and VEGF) in 86 colon cancer patients undergoing curative surgery. □, cancers ($n = 16$) without molecular alterations (high p27, low p53, and VEGF); ▲, cancers ($n = 30$) with one molecular alteration; ■, cancers ($n = 17$) with two molecular alterations; ○. cancers ($n = 23$) with all three molecular alterations.

Table 4 Five-year estimated disease-specific survival (DSS) in 86 colon cancer patients undergoing curative surgery according to Dukes' stage and different molecular patterns

Estimates of the 5-year DSS probability were calculated by the following formula: $S(5\text{ year}, z) = [S_0\ (5\text{ year})]^{\exp(\beta 1\ z1\ +\ \beta 2\ z2\ +\ \beta 3\ z3)}$, where $S(5\text{ year}, z)$ is the 5-year survival probability for covariate vector z; z is a given covariate combination; $z1$, $z2$, and $z3$ are the values of p27, p53, and VEGF codified as negative (0) or positive (1), respectively; $\beta 1$ is the regression coefficient for p27 and equals 2.4186; $\beta 2$ is the regression coefficient for p53 and equals 1.3273; and $\beta 3$ is the regression coefficient for VEGF and equals 0.9100; S_0 is the estimated baseline DSS at 5 years for each stratum of Dukes' stage.

			Dukes' stage		
p27[a]	p53[b]	VEGF[c]	A (n = 21)	B (n = 44)	C + D (n = 18 + 3)
N	N	N	94.5	90.8	79.2
N	N	P	98.5	97.1	94.7
N	P	N	97.8	95.6	92.1
P	N	N	94.7	89.4	81.6
N	P	P	93.7	87.4	78.3
P	N	P	85.1	71.6	54.6
P	P	N	78.2	60.3	39.9
P	P	P	54.4	28.4	10.1

[a] N and P indicate colon cancers with p27 staining >20% or ≤20% of cells, respectively.

[b] N and P indicate colon cancers with p53 staining ≤10% or >10% of cells, respectively.

[c] N and P indicate colon cancers with VEGF staining ≤10% or >10% of cells, respectively.

Analysis Related to DSS in the 86 Patients Subjected to Curative Surgery. The mean follow-up time was 35.5 ± 21 months (range, 8.3–92.6 months; median, 36 months). During this time period, 18 (21%) patients died of disease. Five-year DSS and cumulative risk of cancer death were 67.9 and 38.7%, respectively. The cancer-related mortality rates were not significantly different among groups stratified for age, sex, site, performance status, basal serum carcinoembryonic antigen levels, tumor status, histological grade, tumor growth pattern, tumor size, and postoperative complication rate. On the contrary, the absence of microsatellite instability, the presence of metastatic nodes and/or distant metastases, Dukes' stage higher than B, number of resected and metastatic nodes <2 and >12 were significantly associated with a worse cancer-related death rate (Table 3). The DSS was also strongly related to molecular features. Five-year DSS and cumulative risk of cancer death in high- and low-p27-expression colon cancers were 97 and 38%, and 3 and 96%, respectively. Negative and positive p53 tumors showed 94 and 37% 5-year survival rates, and 6 and 97% cumulative hazard, respectively. VEGF-negative colon cancers had a 5-year DSS and cumulative risk of death of 96 and 3%, compared with 54 and 61% in tumors with VEGF expression, respectively. Tumors with MVC lower and higher than 70 (median value in curatively resected patients) showed a 5-year DSS of 86 and 51%, with a cumulative hazard of cancer death of 15 and 66%, respectively. When we used multivariate analysis, then MVC and other significant variables on univariate analysis, in particular microsatellite stability status, lost their importance. Dukes' stage, low expression of p27, high p53 accumulation, and positive staining for VEGF were demonstrated to be the only covariates independently associated with DSS. After backward elimination, no variables were removed from the model; stepwise regression selected again p27 (P = 0.001), p53 (P = 0.006), VEGF (P = 0.023), and Dukes' stage (P = 0.041) as the best combination of variables to predict DSS (Table 3). Additional insights into the prognostic relevance of molecular patterns was obtained by Cox analysis using different associations of the three molecular markers as a stratification factor. Colon cancers with high p27 expression, low p53 accumulation, and no staining for VEGF showed a 12% hazard of

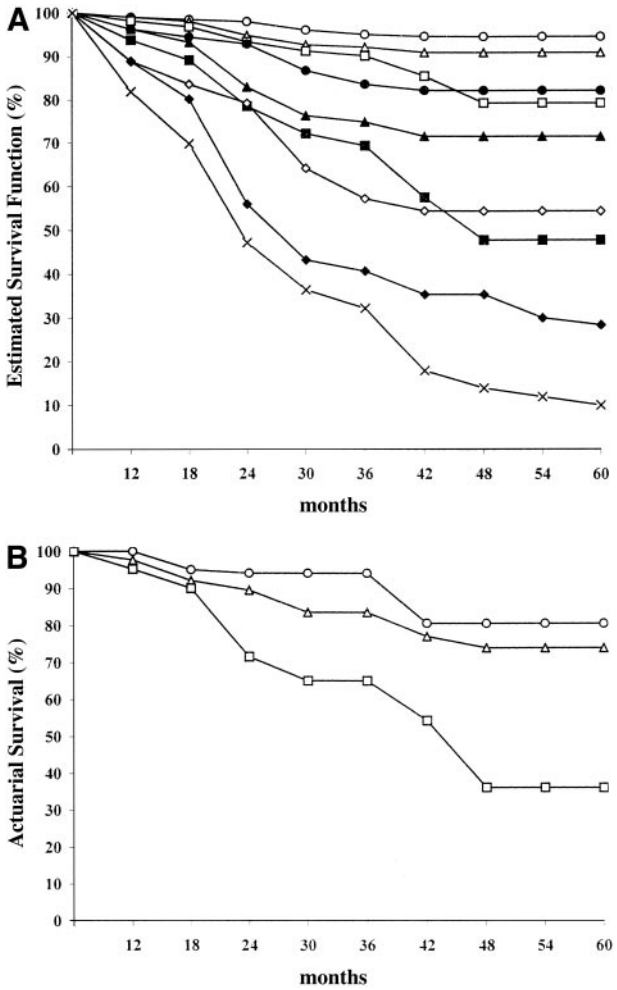

Fig. 2 Estimated 5-year disease-specific survival function stratified for Dukes' stage and different combination of three molecular markers (p27, p53, and VEGF) in 86 colon cancer patients undergoing curative surgery (*A*); for comparison, in *B* is shown the disease-specific survival stratified for Dukes' stages. *A*, ○, Dukes' A cancers without molecular alterations (high p27, low p53, and VEFG); △, Dukes' B cancers without molecular alterations; □, Dukes' C or D cancers without molecular alterations; ●, Dukes' A cancers with one or two molecular alterations; ▲, Dukes' B cancers with one or two molecular alterations; ■, Dukes' C or D cancers with one or two molecular alterations; ◇, Dukes' A cancers with all three molecular alterations (low p27, high p53, and VEGF); ◆, Dukes' B cancers with all three molecular alterations; ×, Dukes' C or D cancers with all three molecular alterations. *B*, ○, Dukes' A cancers; △, Dukes' B cancers; □, Dukes' C and D cancers.

Table 5 Statistical analysis related to disease-free survival and tumor recurrence rate in 86 colon cancer patients undergoing curative surgery

Variable	*n*	TR[a]	NER	χ^b	Cox *P* value	Stepwise multivariate analysis			Dukes' stage, TR/NER		
						Hazard rate	95% CI[c]	*P*	A	B	C/D
p27				0.001	0.006	20.846	18.81–22.87	0.001			
≥20	48	1	47						0/12	1/26	0/9
<20	38	21	17						3/6	9/8	9/3
p53				0.001	0.006	8.120	6.64–9.59	0.003			
≤10	45	2	43						0/10	0/25	2/8
>10	41	20	21						3/8	10/9	7/4
VEGF				0.001	0.036	3.730	2.72–4.73	0.008			
≤10	32	1	31						0/12	1/13	0/6
>10	54	21	33						3/6	9/21	9/6
Dukes' stage											
A	21	3	18								
B	44	10	34	0.087	0.018	2.023	1.45–2.59	0.018			
C + D	21	9	12								

[a] TR, tumor recurrence; NER, no evidence of recurrence; VEGF, vascular endothelial growth factor.
[b] χ^2 test, tumor recurrence *versus* no evidence of recurrence.
[c] 95% confidence interval (CI) of hazard rate of tumor recurrence associated with higher Dukes' stage, positive VEGF immunoreactivity, p53 protein overexpression, and low p27 protein accumulation.

cancer-related death at 42 months; in contrast, colon cancers with low p27 expression, high p53 accumulation, and immunoreactivity for VEGF had an almost 100% risk of death due to tumor in the same time frame (Fig. 1). On the basis of the model generated by the stepwise process, the five-year estimated survival function after potentially curative surgery was evaluated for different combinations of Dukes' stages and molecular markers. As shown in Table 4, the different molecular patterns added strong, independent prognostic information to Dukes' stages. In fact, in Dukes' A, B, and C plus D tumors, the 5-year survival probability declined progressively from 94.5 to 54.4%, from 90.8 to 28.4%, and from 79.2 to 10.1%, respectively, with the increase in the number of molecular alterations. Moreover, it has to be emphasized that, when we stratified for Dukes' stages, colon cancers with more advanced stages but fewer molecular alterations did much better than tumors with earlier stages but unfavorable molecular patterns (Fig. 2). For example, Dukes' A and B colon cancers expressing low p27 and high p53 and VEGF had a 5-year estimated survival function significantly lower than Dukes' C or D tumors without molecular alterations.

Analysis Related to DFS and Tumor Recurrence Rate in the 86 Patients Subjected to Curative Surgery. Twenty-two patients (25.5%) experienced tumor recurrence; 4 are alive without tumor after reoperation. No local recurrences were seen; thus, all of the recurrences were distant metastases and occurred in the liver, and in some instances in the lung and/or bones as well. Mean recurrence time was 18.9 ± 8 months (range, 10–38 months; median, 15 months). In all but five patients (77%), recurrence time was shorter than 2 years. Five-year DFS and cumulative risk of recurrence were 72.5 and 32.1%, respectively. As for cancer-related death rate, the presence of nodal and/or distant metastases, Dukes' C and D stages, absence of microsatellite instability, total number of both resected and metastatic nodes, low p27 expression, p53 accumulation, VEGF immunoreactivity, and MVC >70 were significantly related to a worse DFS rate. However, on Cox' analysis, only Dukes' stage, p27, p53, and VEGF were shown to be independent prognostic factors for DFS. This was confirmed by stepwise multivariate analysis that included only such four covariates in the model best predicting tumor recurrence (Table 5). Molecular markers were very strong prognostic indicators of tumor recurrence. Forty-two-month cumulative hazards of tumor relapse in colon cancer with low p27 accumulation, p53 overexpression, and positive VEGF immunoreactivity were 96.9, 86.4, and 66.4%, respectively. These percentages were significantly higher than those predicted by the conventional system based on Dukes' stage, because Dukes' A, B, and C/D colon cancers

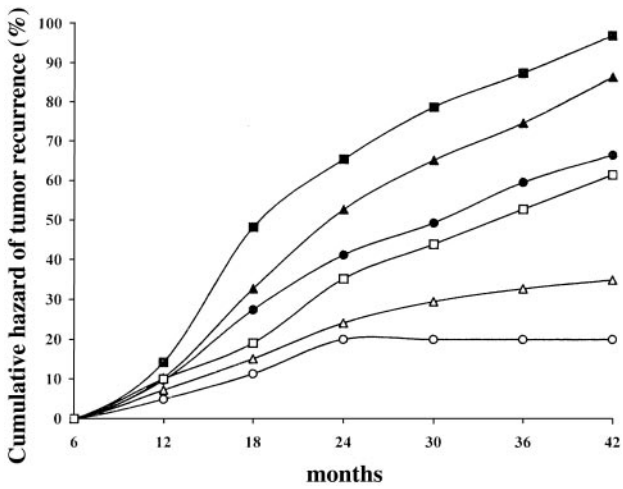

Fig. 3 Multivariate cumulative hazard of tumor recurrence rate according to different molecular markers and Dukes' stages in 86 colon cancer patients undergoing curative surgery. ■, cancers with p27 protein downregulation; ▲, cancers with high p53 protein expression; ●, cancers with high VEGF immunoreactivity; □, Dukes' C/D cancers; △, B cancers; ○, Dukes' A cancers

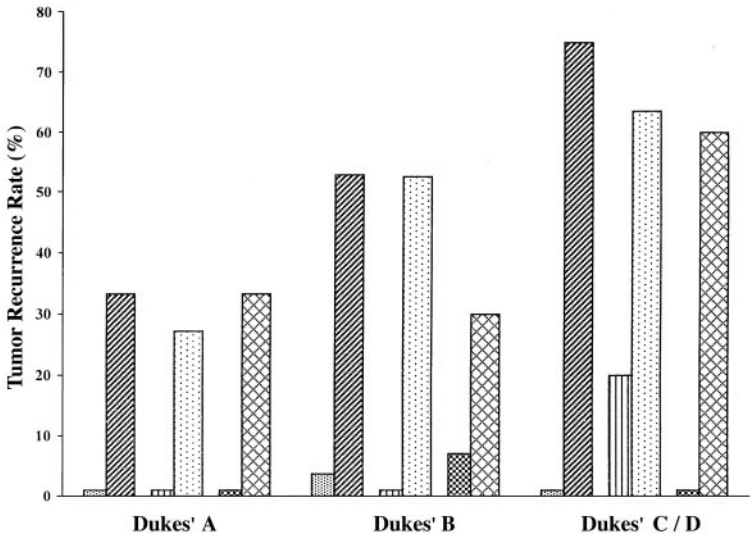

Fig. 4 Tumor recurrence rate in 86 colon cancer patients undergoing curative surgery stratified for Dukes' stage and different molecular profiles. ▨, high p27 protein accumulation; ▨, low p27 protein accumulation; ▥, low p53 protein overexpression; ▨, high p53 protein overexpression; ▩, negative VEGF immunoreactivity; ▩, positive VEGF immunoreactivity.

showed a risk of relapse after curative surgery of 20, 34.9, and 64.4%, respectively (Fig. 3). Moreover, stratifying for Dukes' stage and molecular profile, clear and significant discrepancies appeared in and among different Dukes' stages. Indeed, earlier-stage colon cancers with worse molecular patterns showed a significantly higher tumor recurrence rate than more advanced-stage colon cancers with no molecular alterations (Fig. 4).

DISCUSSION

The gold standard for clinical outcome of most cancers has been the clinical and pathological staging of the tumor after surgery. However, current methods to predict outcome for patients with curatively colon cancer are not ideal (18, 26, 27, 32). Recently, many studies reported that combinations of molecular markers could be equivalent to pathological or clinical staging in predicting clinical outcome. Moreover, it has been suggested that the knowledge of molecular features which determine the behavior of individual colon tumors may represent a fundamental step to identify high-risk categories of patients, thus allowing modulation of cancer treatment options (1–3, 23, 30, 33). Of interest, some reports have pointed out significant correlations between DNA microsatellite instability and low expression of p53 and VEGF, suggesting a molecular basis for the clinical observation that microsatellite instability colon cancers have a more favorable prognosis (34). However, other studies have been unable to find any correlation between tumor molecular features and clinical outcome, warranting additional molecular and clinicopathological investigations to further define the relevance of molecular markers for the prognosis of colon cancer patients (14, 15, 19, 25, 32).

In this study, we have analyzed, by immunohistochemical methods, four indicators involved in cell cycle regulation (p27 and p53), apoptosis (p53 and p27), and tumor neoangiogenesis (p53, VEGF, and MVC). An important step was to compare immunohistochemical staining in normal and cancer colon cells, respectively. All normal-appearing mucosal colon cells showed intense nuclear staining for p27 protein, with $<10\%$ p53 and VEGF expression; moreover, MVC was significantly lower than in tumor samples. Our data are consistent with other studies confirming the assumption that the molecular profile in nonproliferative colon mucosal cells is quite well established (3, 10, 15, 28). Also, our immunohistochemistry analysis confirms other studies suggesting that, inside the tumor, the molecular profile of colon cancers has a regular distribution (13).

Molecular markers showed a strong correlation with each other and were good indicators of the likelihood to perform curative surgery, and p27 down-regulation and VEGF overexpression significantly correlated with Dukes' stages and the presence of distant metastases, indicating that advanced colon cancers were associated with more evident molecular alterations. The absence of correlation between p53 up-regulation and more aggressive colon cancer behavior confirms the hypothesis that p53 alteration is primary in colon carcinogenesis (*i.e.*, progression from adenoma to carcinoma) regardless of tumor stage (17, 35).

The correlation between the presence of distant metastases and expression of molecular markers is noteworthy. Because VEGF plays a crucial role in neoangiogenesis, it is not surprising that its overexpression is associated with a higher metastatic rate (23). However, a higher MVC was surprisingly not associated with it, suggesting either that MVC may be a method with poor accuracy or that tumor aggressiveness is independent of MVC (24, 25). p27 down-regulation and metastatic rate were also well correlated. This confirms the hypothesis that the p27 gene, as well as cell cycle, regulates the mechanisms of cell adhesion, and its down-regulation confers on tumor cells the ability to grow in an environment of altered intercellular adhesion or extracellular matrix, two processes that facilitate metastases (12–14).

In this study, molecular patterns strongly correlated with DSS and DFS in curatively treated colon cancer patients. Of interest, because no correlation was found between any molecular marker and DNA microsatellite stability status, and the latter was not an independent prognostic indicator of DSS and DFS on multivariate analysis, these results suggest that, in our

series, the prognostic significance of the molecular markers was not associated with DNA microsatellite status.

p27, p53, and VEGF, together with Dukes' stage, were the best combination of variables predicting long-term survival and recurrence rate. However, relevant discrepancies in clinical outcome were observed among different Dukes' stages. On the contrary, the prognostic significance of molecular markers was more accurate. Indeed, the greater the number of molecular alterations, the higher the hazard rate of cancer-related death. Furthermore, the 5-year estimated survival function after curative surgery decreased markedly as the number of molecular alterations increased through different Dukes' stages. Moreover, each molecular marker predicted recurrence rate much better than the conventional system based on Dukes' stage. Colon cancers without molecular alterations showed a very slight increase in recurrence rates with worsening Dukes' stages. In contrast, the presence of any unfavorable molecular pattern determined a significant higher recurrence rate. Thus, the determination of molecular alterations may help explain the different outcome observed in colon cancer patients with the same Dukes' stage as well as the longer survival in patients with more advanced colon tumors. In this respect, a simple prognostic model including the molecular pattern and Dukes' stage can be proposed to identify subsets of patients with a very wide range of prognostic estimates, *i.e.*, with a 5-year probability of death spanning from 5.5% with the best combination of the prognostic factors to approximately 90% with the worst combination. This finding is very interesting from a clinical standpoint, because a similar model might be very useful in selecting among different postsurgical treatment strategies and in improving the counseling of patients.

In conclusion, the determination of molecular markers by immunohistochemistry seems to be, in colon cancer, an easy, reliable, and useful method to gain insight into tumor behavior and select treatment modality. In our series, p27, p53, and VEGF were the strongest independent prognostic indicators for patients with colon cancer treated with curative surgery; in contrast, MVC requires additional investigations to better elucidate its role. Individual molecular markers, or better, a combination of the three analyzed molecular markers, seem to be equivalent of, or even better than, conventional clinicopathological staging procedures for prediction of the outcome and recurrence rate in curatively treated colon cancer patients. In addition, molecular markers can be used in conjunction with clinical and pathological staging to provide a stronger indicator of clinical outcome than with staging alone. In agreement with other investigators (33), we suggest adding "molecular staging" to conventional staging procedures to better select therapeutic options and predict clinical outcome in colon cancer patients.

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