Comparison of microsatellite instability and chromosomal instability in predicting survival of patients with T3N0 colorectal cancer

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Background. *At least 2 apparently independent mechanisms, microsatellite instability (MSI) and chromosomal instability, are implicated in colorectal tumorigenesis. Their respective roles in predicting clinical outcomes of patients with T3N0 colorectal cancer remain unknown.*
Methods. *Eighty-eight patients with a sporadic T3N0 colon or rectal adenocarcinoma were followed up for a median of 67 months. For chromosomal instability analysis, Ki-ras mutations were determined by single-strand polymerase chain reaction, and p53 protein staining was studied by immunohistochemistry. For MSI analysis, DNA was amplified by polymerase chain reaction at 7 microsatellite targets (BAT25, BAT26, D17S250, D2S123, D5S346, transforming growth factor receptor II, and BAX).*
Results. *Overall 5-year survival rate was 72%. p53 protein nuclear staining was detected in 39 patients (44%), and MSI was detected in 21 patients (24%). MSI correlated with proximal location (*P < *.001) and mucinous content (*P < *.001). In a multivariate analysis, p53 protein expression carried a significant risk of death (relative risk = 4.0, 95% CI = 1.6 to 10.1,* P = *.004). By comparison, MSI was not a statistically significant prognostic factor for survival in this group (relative risk = 2.2, 95% CI = 0.6 to 7.3,* P = *.21).*
Conclusions. *p53 protein overexpression provides better prognostic discrimination than MSI in predicting survival of patients with T3N0 colorectal cancer. Although MSI is associated with specific clinicopathologic parameters, it did not predict overall survival in this group. Assessment of p53 protein expression by immunocytochemistry provides a simple means to identify a subset of T3N0 patients with a 4-times increased risk for death. (Surgery 2002;131:190-7.)*

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TWO MAJOR mechanisms of genomic instability have been identified in sporadic colorectal cancer progression. The first, known as chromosomal instability (CIN), results from a series of genetic changes that involve the activation of oncogenes, such as Ki-*ras*, and inactivation of tumor-suppressor genes, such as TP53 and APC.[1,2] The second, known as microsatellite instability (MSI), was initially described in association with hereditary nonpolyposis colorectal cancer, but it is also found in 12% to 15% of sporadic colorectal cancer cases.[3,4] In sporadic colorectal cancer, MSI results from somatic (acquired) inactivation of the DNA mismatch repair gene MLH1 by hypermethylation of its promoter, and secondary mutation of genes with coding microsatellites, such as transforming growth factor receptor II and BAX.[5] Colorectal cancer with MSI has a distinct phenotype, characterized by mucinous growth, proximal location, poor histologic differentiation, and, paradoxically, by a more favorable stage distribution.[6] Two distinct MSI tumor phenotypes have been described: MSI-H (MSI at >30% of microsatellites examined) and MSI-L (<30% microsatellites examined).[7] MSI-H tumors are associated with inactivation of DNA mismatch repair function, whereas MSI-L tumors behave like tumors with CIN.

The initial description of MSI also described a trend toward prolonged survival for these patients, and since then, MSI has been repeatedly reported

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as a favorable prognostic marker.[8] However, in most studies, the survival advantage associated with MSI was not independent from the stage of the disease. In other words, TNM classification remained the best indicator for prognosis when a multivariate analysis was performed. In addition, some investigators considered only selected categories of patients (<50 years of age) and used various definitions of MSI.[9-11] Finally, few studies have correlated the prognostic significance of MSI with analyses of CIN, such as loss of heterozygosity on 17p or 18q, which have been associated with an adverse clinical outcome in colorectal cancer.[12-14]

Supplementation of TNM classification by genetic analysis is particularly relevant in node-negative colorectal cancer. Patients with T3-T4, N0, and M0 (Dukes' B) tumors represent roughly 50% of all patients with colorectal cancer and have a 5-year survival rate of 60% to 75% with operation alone.[15] Although an improvement in survival rate has been established for patients with Dukes' C colon cancer who received 5-fluorouracil and levamisole, this has not been the case for patients with Dukes' B cancer.[16] Thus, to date, there is no indication for systemic adjuvant chemotherapy in this group.[17] Altogether, these data suggest that 15% to 20% of all patients with colorectal cancer will eventually die of metastatic disease without having been considered initially as candidates for systemic adjuvant chemotherapy. However, identifying this subgroup of patients at high risk has proven difficult despite the use of sophisticated techniques that require a high degree of expertise.[18,19]

Because colorectal tumorigenesis progresses through a sequential multistep process of molecular changes, it is logical to hypothesize that evaluating multiple markers rather than a single marker in isolation will more accurately predict tumor behavior. In addition, the respective roles of MSI and CIN in predicting clinical outcome of colorectal cancer need to be assessed in a non-selected patient population with uniform treatment groups. Therefore, this study was conducted to compare the prognostic significance of the 2 known mechanisms of genomic instability in a homogeneous cohort of patients with T3N0 colorectal cancer followed up for more than 5 years.

PATIENTS AND METHODS

Patients. Eighty-eight samples of T3N0 sporadic colorectal carcinoma were available for analysis. Tumor specimens were obtained from consecutive surgical resections performed electively at the Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, between 1984 and 1989. This group was part of a larger cohort of patients with Dukes' B cancer that were previously studied.[20] The 1984 to 1989 period was chosen because, at that time, no preoperative radiotherapy was administered to patients with lymph node-negative rectal cancer at our institution. Therefore, downstaging of patients with primitive Dukes' C cancer due to preoperative radiotherapy was not expected. All patients included in this study received curative (R0) surgical resection only and were considered a therapeutically homogeneous group. Tumor stage was based on clinical evaluation including abdominal exploration at laparotomy, preoperative chest radiography, and pathologic analysis of surgical specimens.

Criteria for exclusion were emergency procedures; perforated carcinomas; history of hereditary non-polyposis colorectal cancer syndrome; carcinomas associated with inflammatory bowel disease; or presence of another extracolic carcinoma at the time of diagnosis or during the follow-up period. Finally, patients who died in the immediate postoperative period (<30 days after the operation) were excluded from analysis.

Records were retrieved and the following variables were analyzed for prognosis: demographics (age, gender), tumor localization (proximal vs distal colon or rectum), degree of histologic tumor differentiation, and mucinous content. Follow-up was carried out through routine visits at our Outpatient Oncological Clinic. Serum carcinoembryonary antigen levels were assessed every 3 months during the first 2 years after the operation and every 6 months thereafter. All patients received annual colonoscopy and chest x-ray films, and patients with rising serum carcino embryonary antigen levels or clinical findings suggestive of tumor recurrence received abdominal computed tomography scans or liver ultrasonography. Whenever possible, confirmation of data was obtained through interviews with the patients or their physicians.

All samples were reassessed for confirmation of diagnosis. Slides of 88 patients were reviewed by 2 pathologists (P. C., H. B.). For every patient, 1 paraffin block with both tumor tissue and normal mucosa was selected for the detection of p53 protein expression by immunohistochemistry. For K-*ras* mutation analysis, DNA was extracted from paraffin-embedded tissue. Based on the assumption that colorectal adenocarcinomas may be genetically heterogeneous, 2 different tumor samples were analyzed in each patient. One of the samples was also used for p53 immunohistochemistry. DNA and immunohistochemical analyses were

interpreted by pathologists and gastroenterologists, who did not participate in patients' care and were blinded to clinical outcome.

Immunohistochemistry. Four μm-thick tissue sections were mounted on aminopropylmethoxysilane-coated glass slides, deparaffinized in xylol, taken through to absolute alcohol, blocked for endogenous peroxydase with 1% hydrogen peroxide in methanol (45 minutes), and rehydrated through graded alcohols. They were subjected to heating in a microwave oven for 15 minutes in 10 mmol/L of citrate buffer (pH 6.0) and rinsed in TBS (Tris 0.05 mol/L, sodium chloride 0.9%, pH 7.6). The tissue sections were incubated for 10 minutes in normal goat serum (Pel-Freez Biologicals, Rogers, Ark) diluted 1:30 in TBS to reduce nonspecific binding. After incubation (30 minutes) of 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 minutes) with goat anti-mouse immunoglobulins (Sternberger, Baltimore, Md), diluted 1:100 in TBS-nfdm, and, with PAP-complex, diluted 1:600 in TBS-nfdm. Peroxydase activity was revealed with 5,5-diaminobenzidine as chromogen, and the sections were counterstained in Mayer's acid-free hematoxylin. 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 semiquantitatively by 3 observers and, according to the percentage of positive tumor nuclei, scored as follows: (0) for tumors showing less than 10% of immunostained nuclei; (+) for tumors showing 10% to 50% of immunoreactive nuclei; and (++) for tumors with nuclear immunoreactivity in more than 50% of tumor cells.

Detection of Ki-*ras* mutations by non-radioactive single strand conformation polymorphisms. Tumor tissue was dissected from paraffin blocks with a sterile scalpel. After deparaffinization in xylene and proteinase K digestion, the DNA was extracted with phenol and a combination of phenol and chloroform and precipited with ethanol.[21] Ki-*ras* exons 1 and 2 and p53 exons 5 to 8 were amplified separately by polymerase chain reaction (PCR). The PCR reaction was carried out for 35 cycles with the following amplification profile: denaturation at 94°C for 30 seconds, annealing at 54°C to 60°C for 45 seconds, and extension at 73°C for 45 seconds. Correct amplification was controlled by electrophoresis on a 2% agarose gel. Five to 40 ng of PCR product were denatured in 10 μL of 50 mmol/L of sodium hydroxide and 1 mmol/L of ethylenediamine tetraacetic acid at 50°C for 10 minutes. These conditions allow for an almost complete denaturation of the DNA. After the addition of 1.5 μL of formamide dye, the samples were immediately analyzed on a 12% MDE gel (FMC BioProducts, Rockland, Me) solution. Electrophoresis was performed at 20°C on a vertical gel in a Hoeffer SE600 apparatus, at 20 V/cm in 0.5 × TBE for about 4 hours. The gels were stained with SYBY green II (FMC BioProducts, Rockland, Me) and visualized under ultraviolet light using a CCD camera. This technique allows the detection of at least 10 mutated alleles among 100 wild type alleles. 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.

Tumors: microsatellite and loss of heterozygosity assays. We used the reference panel of microsatellite primers recommended for colorectal cancer specimens to determine the presence of microsatellite instability.[22] These included the microsatellite markers BAT25, BAT26, D5S346 (APC), D2S123 (hMSH2), and D17S250 (p53). Each primer pair was optimized for efficient amplification. One primer from each pair was radio-end-labeled with ^{32}P or ^{33}P in a reaction containing the primer, kinase buffer, T4 polynucleotide kinase, and $\gamma^{32}P$ or $\gamma^{33}P$ adenosine triphosphate. PCR was undertaken on the microdissected template DNA in a reaction containing 0.125 pmol each of the endlabeled and "cold" primers, 0.25 U of Taq DNA polymerase, 40 mmol/L of deoxyribonucleoside triphosphate stock solution, and a final concentration of 1.5 to 2.0 mmol/L magnesium. PCR products were denatured in 95% formamide and electrophoresed on a 6% polyacrylamide gel that contained 7.5 mol/L urea. The gels were then dried and exposed by autoradiography to x-ray film. MSI was defined as a novel allele in the tumor DNA compared with the normal (non-tumor) DNA. MSI was defined as instability at 2 or more loci (MSI-H). MSI-L was defined by the presence of instability at 1 loci only.

Statistical analysis. The primary endpoint for this analysis was overall survival, defined as time from the operation to death. Statistical analyses were carried out by means of the software package SAS on a UNIX system. Quantitative data were expressed as mean ± SD. Group comparisons were made with the use of chi-square tests for categorical variables, *t* tests for continuous variables, and log-rank tests for the time-to-death variables. Estimated

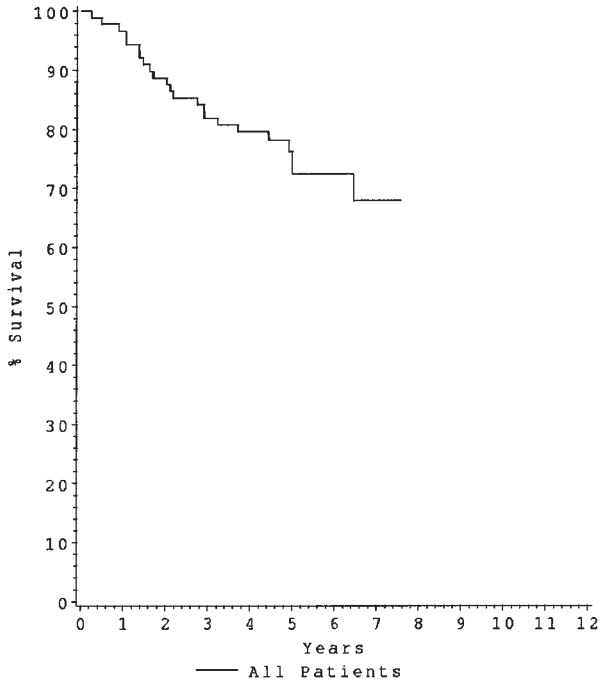

Fig 1. Kaplan-Meier survival curve for the whole group.

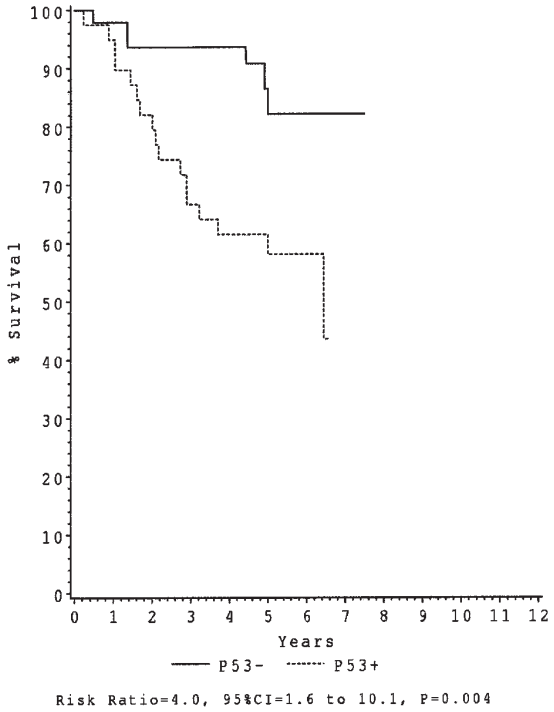

Fig 2. Kaplan-Meier survival curve according to p53 protein expression.

hazard ratios of death (or of first event), with respect to the chosen reference group, their 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 hazard ratios 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: age of the patients; gender; location of the tumor (proximal vs distal vs rectal); histologic grade; mucinous component; p53 protein overexpression [0 vs (+) or (++)]; p53 mutation (absent vs present); Ki-*ras* mutation (absent vs present); and MSI-H (present vs absent). Forward selection was used to build the final model. All reported *P* values are for 2-sided tests. An α-level of .05 was used as the cut-point for statistical significance.

RESULTS

The median follow-up time was 67 months. There were 46 men and 42 women. The mean age was 70 ± 11 years. During the period of observation, 23 patients died. For the whole group, the overall 5-year survival rate was 72% (Fig 1).

CIN tumors. Abnormal p53 protein expression (nuclear staining present in >10% of cancer cells), visualized with inactivation of p53, was detected in 39 (45%) patients. p53 positive tumors were more likely to be located distally to the splenic flexure ($P < .001$), to be well or moderately differentiated ($P = .03$), and to be non-mucinous ($P < .001$). In addition, 95% (37 out of 39) of p53 positive tumors were MSI negative ($P < .001$). The correlation between p53 protein expression and the clinicopathologic features of the patients are summarized in Table I.

MSI tumors. Twenty-one tumors (24%) exhibited microsatellite instability at 2 or more loci, and were considered as MSI-H. Two patients exhibited MSI at 1 satellite and were considered to be MSI-L. Seven tumors exhibited instability at 6 or more loci; 7 tumors showed instability at 5 loci; 3 tumors had instability at 4 loci; and 4 tumors had instability at 2 or 3 loci. Markers BAT26 (17 patients, 81%) and BAT25 (16 patients, 76%) exhibited the highest levels of sensitivity for tumors with MSI-H phenotype. Patients with MSI-H tumors showed a trend toward older age ($P = .09$). MSI-H tumors were more likely to be located proximally to the splenic flexure ($P < .001$) and to be poorly differentiated ($P < .001$). Nineteen out of 21 MSI-H tumors (90%) were negative for p53 protein ($P < .001$). Table II summarizes the correlation between MSI status and the clinicopathologic features of the patients. None of the MSI-H tumors were located within the rectum.

Survival according to p53 protein expression. We first considered 3 groups for survival analysis. Two groups showing positive nuclear expression of p53 protein in more than 10% and more than 50%

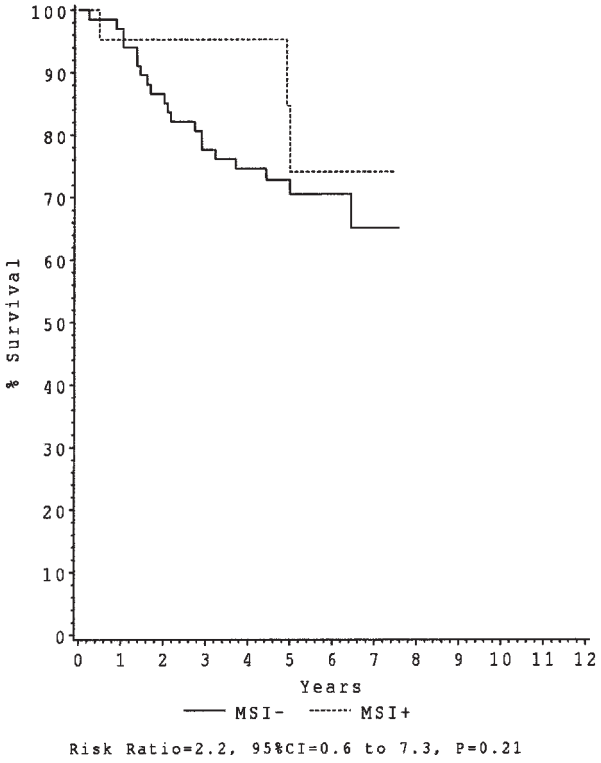

Fig 3. Kaplan-Meier survival curve according to MSI status.

of tumor cells, respectively, did not differ in their survival characteristics and were subsequently considered together and compared with the group with absence of expression of p53 protein (p53-negative group, defined as protein expression in less than 10% of tumor cells). In multivariate analysis, p53 protein expression carried a significant risk of death (RR = 4.0, 95% CI = 1.6 to 10.1, $P = .004$) (Fig 2).

Survival according to MSI-H. Although patients with MSI-H tumors had a slightly better outcome, this difference did not reach statistical significance (RR = 2.2, 95% CI = 0.6 to 7.3, $P = .21$) (Fig 3). However, the Kaplan-Meier survival curves, when compared between groups, demonstrate a different pattern, with no death observed in the MSI-H group between 6 months and 62 months after the operation. Had the follow-up been interrupted at 5 years, univariate analysis of survival would have demonstrated a statistically significant difference between both groups (log-rank test, $P < .05$).

Ki-*ras* mutation and MSI. Presence of a Ki-*ras* mutation was detected in 29 (33%) patients. Most mutations occurred on codon 12 and were of the GAT or GTT type. Four (19%) MSI-H tumors had an associated Ki-*ras* mutation.

DISCUSSION

Our data show that one fourth of T3N0 (Dukes' B2) colorectal tumors are characterized by microsatellite instability. When considering only proximal T3N0 tumors, more than 50% of them are MSI-H. Less than 10% of tumors located distally to the splenic flexure (part of the colorectum derived from the hindgut) are MSI-H. A significant negative association was found between MSI and p53 protein overexpression. In this homogenous group of patients treated with an operation alone and followed for more than 6 years, MSI was not associated with a favorable outcome. Conversely, overexpression of the p53 protein was clearly a predictor of decreased overall survival. Finally, a small subset of tumors exhibited features common to MSI and loss of heterozygosity (Ki-ras mutation or p53 protein overexpression).

A number of investigations have dealt with the prognostic significance of MSI in colorectal cancer.[23-25] Surprisingly, few studies have focused on MSI status in Dukes' B patients, despite the fact that, in this group, identification of a subset of patients with high-risk disease may result in improved clinical management. The apparent contradiction in the current literature regarding the prognostic value of MSI in colorectal cancer is, for the most part, due to the fact that MSI-H tumors are more prevalent in early stage (node-negative) cancers.[3] The percentage of MSI among stage II tumors is 20% to 25%, while it is 10% to 12% in stage III and virtually absent in Stage IV colorectal cancer.[11] The survival advantage apparently conferred by MSI disappears when considering a patient population with a similar tumor stage. In addition, MSI-H patients are generally older and therefore more at risk for dying of non cancer-related causes. Thus, MSI-H patients may have a better cancer-specific, but not a better overall, survival rate. In accordance with others, our results suggest that MSI has limited prognostic value when considering overall survival in a cohort of patients with similar tumor stages.[26,27]

Most sporadic colorectal cancers, are from the CIN phenotype, and exhibit major abnormalities in chromosome numbers, structure, or both, as well as evidence for sensitivity to 5-fluorouracil relative to MSI-H cells.[28] In accordance with Leahy et al,[29] our data demonstrate that p53 abnormalities detected at the protein level by immunocytochemistry provide better prognostic discrimination than those detected by non-radioactive single strand conformation polymorphisms analysis. Because the monoclonal antibody DO-7 reacts with both the wild-type and mutant types of the p53 protein, it is likely that, in these patients, positive immunostaining results from the accumulation of a mutated but stabilized protein. Our results also agree with previ-

Table I. Relationship between p53 protein expression and clinicopathologic variables in T3N0 colorectal cancers

Variable	*Level*	*p53– (%)*	*p53+ (%)*	P *value*
All patients (N = 86*)		47 (55%)	39 (45%)	—
Gender	Male	22 (47%)	24 (62%)	.17
	Female	25 (53%)	15 (38%)	
Age (mean ± SD)		(69.3 ± 12.0)	(69.7 ± 9.3)	.86
Location	Proximal	25 (53%)	5 (13%)	< .001
	Distal + Rectal	22 (47%)	34 (87%)	
Differentiation	Well + Moderate	37 (79%)	37 (95%)	.03
	Poor	10 (21%)	2 (5%)	
Mucinous	Yes	24 (51%)	3 (8%)	< .001
	No	23 (49%)	36 (92%)	
MSI	+	19 (40%)	2 (5%)	< .001
	–	28 (60%)	37 (95%)	
Ki-*ras*	Wild-type	29 (62%)	30 (77%)	.13
	Mutated	18 (38%)	9 (23%)	
% Survival	1-year	98%	95%	
	3-year	94%	67%	.004
	5-year	91%	62%	

*Two patients had missing values for p53.

Table II. Relationship between MSI status and clinicopathologic variables in T3N0 colorectal cancers

Variable	*Level*	*MSI-H (%)*	*MSI-L/MSI (%)*	P *value*
All patients (N = 88)		21 (24%)	67 (76%)	—
Gender	Male	12 (57%)	34 (51%)	.61
	Female	9 (43%)	33 (49%)	
Age (mean ± SD)		(73.0 ± 9.4)	(68.5 ± 10.9)	.09
Location	Proximal	16 (76%)	14 (21%)	< .001
	Distal + Rectal	5 (24%)	53 (79%)	
Differentiation	Well + Moderate	16 (76%)	60 (90%)	.12
	Poor	5 (24%)	7 (10%)	
Mucinous	Yes	14 (67%)	13 (19%)	< .001
	No	7 (33%)	54 (81%)	
p53	+	2 (10%)	37 (55%)	< .001
	–	19 (90%)	28 (42%)	
Ki-*ras*	Wild-type	17 (81%)	44 (66%)	.19
	Mutated	4 (19%)	23 (34%)	
% Survival	1-year	95%	97%	
	3-year	95%	78%	.21
	5-year	95%	73%	

ous series showing that p53 protein expression is a determinant of poor clinical outcome in colorectal cancer.[30-32] However, other genetic features found in CIN tumors, such as deletions in chromosomes 18q or 8p, may actually provide better prognostic information.[14,33]

Our data, however, do not provide support for an intriguing hypothesis recently formulated: the existence of a third molecular pathway in colorectal carcinogenesis. Some authors have characterized a subtype of colorectal cancer combining features of the suppressor and mild mutator (MSI-L) pathways.[34] Others have identified a subset of sporadic colorectal cancers with evidence neither for chromosomal, nor for microsatellite instability.[35] In our series, there were 4 (19%) MSI-H tumors that exhibited Ki-*ras* mutations; in addition, 10 out of 88 tumor specimens were simultaneously negative for MSI and CIN.

The clinical application of molecular genetics in patients with colorectal cancer is in development. The application of such techniques can be expected eventually to help the clinician in stratifying adjuvant therapy decisions and supplementing standard clinicopathologic staging. As with most components of research, there was limited control

over the nature and quality of measurements taken. Selection bias is inherent in all non-randomized studies. That being said, the inclusion criteria were designed to identify a homogeneous group of patients to represent the target population. These criteria were purposely not too strict, so as to balance the need for homogeneity with that for generalizability. The smaller than ideal sample size was of particular concern when no statistically significant difference was found. In these cases, such as that of MSI, it is impossible to determine whether there was adequate evidence to support the conclusion of "no difference" or if, rather, there was insufficient power to detect a clinically important difference. Non-significant results were interpreted with caution, and further studies are needed for more conclusive findings.

In summary, MSI and CNI represent 2 fairly discrete molecular pathways in colorectal cancer development. Together, these 2 pathways account for 90% of all T3N0 tumors. More than 50% of the tumors located proximally to the splenic flexure showed microsatellite instability. In this series of non-selected T3N0 tumors, MSI-H phenotype showed a trend toward improved survival. However, abnormal expression of the p53 protein was a strong predictor of poor outcome, and provided better prognostic discrimination than MSI status.

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