p53 Mutations in L3-Loop Zinc-binding Domain, DNA-Ploidy, and S Phase Fraction Are Independent Prognostic Indicators in Colorectal Cancer : A Prospective Study with a Five-Year Follow-Up

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p53 Mutations in L3-Loop Zinc-binding Domain, DNA-Ploidy, and S Phase Fraction Are Independent Prognostic Indicators in Colorectal Cancer: A Prospective Study with a Five-Year Follow-Up[1]

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Abstract

***p53* gene alterations are among the most common events observed in colorectal cancer, and are accompanied frequently by DNA aneuploidy and high proliferative activity. The prognostic significance of such mutations remains controversial. We prospectively evaluated the prognostic significance of *p53* mutations, DNA-ploidy, and S phase fraction (SPF) in a consecutive series of 160 colorectal cancer patients (median follow-up 71 months). Tumor DNA was screened for *p53* mutations by PCR/single-strand conformational polymorphism/sequencing. DNA-ploidy and SPF were assessed by DNA flow cytometry. *p53* mutations were detected in 68 of 160 (42.5%) cases. In 56% (38 of 68) of these, *p53* mutations were found in conserved areas of the gene and in 44% (30 of 68 cases) outside the conserved regions. Eighteen of the 68 cases (26%) had mutations in the L3 loop, 11 of 68 (16%) in the L1 loop-sheet-α helix motif, and 39 of 68 (58%) outside L3 and loop-sheet-α helix. Seventy-five percent of the cases (120 of 160) showed DNA aneuploidy, whereas 18% of these (22 of 120) were multiclonal. The major independent predictors for both disease relapse and death were advanced Dukes' stage, *p53* mutations affecting L3 loop, DNA-aneuploid tumors, and high SPF (>18.5%). Our results show that mutations in L3 functional domain, more than any mutations, are important biological indicators to predict the outcome of patients indicating that these mutations have biological relevance in terms of colorectal cancer disease course.**

Introduction

The classic adenoma-carcinoma sequence in CRC[4] results from an accumulation of somatic genetic changes responsible for a progressively more aggressive cell behavior (1). These alterations, which may range from single nucleotide substitutions, deletions and insertions, to rearrangements, amplifications, and loss of genetic material often resulting in gross variations in cell DNA content (DNA-ploidy), are ultimately responsible for the activation of proto-oncogenes and the inactivation of tumor suppressor genes leading to uncontrolled proliferative activity. It has been suggested that characterization of the genomic abnormalities acquired during colorectal tumorigenesis might provide additional prognostic information. However, although multiclonality would seem to be a frequent indicator of worse clinical outcome (2, 3), the available data regarding the prognostic significance of DNA-ploidy in CRCs are still controversial (4), whereas the prognostic value of SPF as a measure of the proliferative activity of the cancer cells is more clearly established (5). Mutations of the *p53* gene have been observed in >50% of CRCs, as well as in other neoplastic diseases, mostly in the protein core domain (6). The integrity of this region, which includes 4 of the 5 domains highly conserved among species (area II, codons 112–141; area III, 171–181; area IV, 234–258; and area V, 271–286), appears essential for the DNA-binding and transcriptional regulatory activity of p53 (7), which in response to DNA damage or other forms of cellular stress activates and coordinates multiple pathways leading to cell cycle arrest, in G_1-S, G_2-M, or to apoptosis (8). Within this region, several structural domains with distinct roles have been identified: the L2 loop (codons 163–195), required for the folding and stabilization of the central part of the protein; the L3 loop (codons 236–251), and the LSH motif (codons 273–286) within which at least two residues (241, 248 and 273, 280, respectively) contact the DNA directly (Ref. 9; Fig. 1). Most studies performed to evaluate the prognostic significance of p53 mutations in CRCs have taken into account any mutation of the gene, usually on analysis of the region spanning exons 5–8 (codons 130–286), and the results obtained thus far have proved to be rather discordant (6), with only some of them indicating an influence of p53 mutations on DFS and OS rate (10, 11). However, as suggested by Borresen-Dale *et al.*

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[4] The abbreviations used are: CRC, colorectal carcinoma; DFS, disease-free survival; DI, DNA index; HR, hazard ratio; LSH, L1 loop-sheet-α helix; L2 loop; L3 loop; M, mucosa; OS, overall survival; SPF, S phase fraction; SSCP, single-strand conformation polymorphism; T, tumor; Zn-BD, zinc-binding domain.

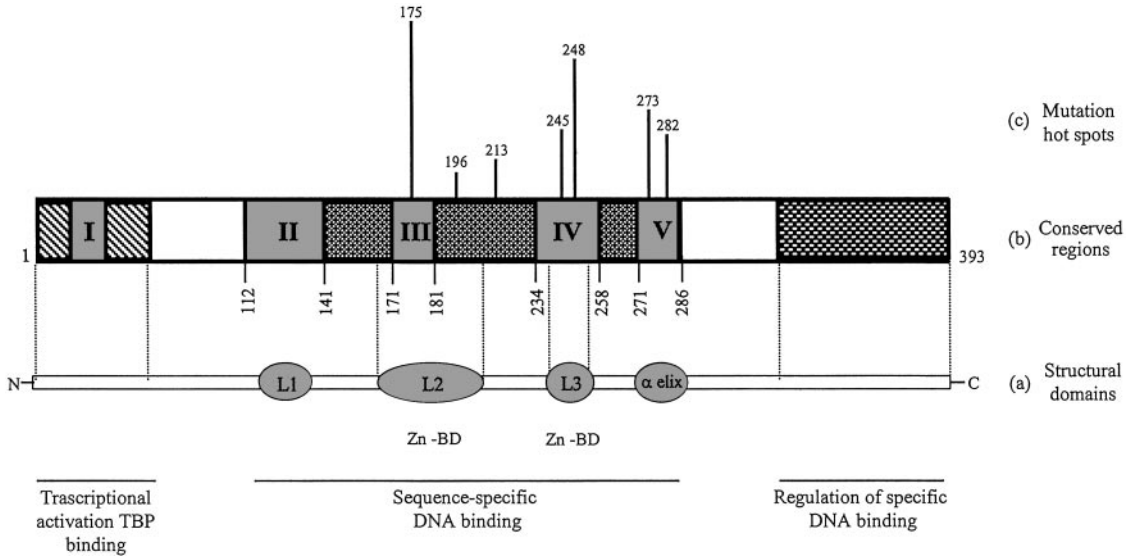

Fig. 1. Schematic representation of the p53 protein structural domains (*a*), highly conserved regions of *p53* gene (*b*), and mutation hot spots in CRC as reported in literature (*c*).

(12), p53 mutations affecting regions that are important for particular functions of the protein may have a stronger prognostic impact. Moreover, p53 mutations in evolutionary conserved regions were found to be associated with tumors that are not only biologically more aggressive (13), but probably also chemoresistant (14). Support to these findings is provided by the more recent report by Skaug *et al.* (15) that mutations within specific domains (L2 loop, L3 loop, and LSH motif) of p53 are associated with a more aggressive phenotype, presumably because these mutations have a particularly strong negative effect on the biological activity of the protein.

Therefore, the aim of our prospective study was to assess in 160 patients who had undergone curative surgical resection for primary CRC the possible association between any or specific p53 mutations, DNA-ploidy, SPF, and the clinicopathological variables, and, furthermore, to evaluate the prognostic significance of the biomolecular variables themselves.

Materials and Methods

Patient Features. A prospective study was performed on paired tumor and normal colon tissue samples from a consecutive series of 160 patients undergoing resective surgery for primary operable CRC at a single institution (Department of Oncology, University of Palermo, Palermo, Italy) from January 1988 to December 1992.

Elegibility criteria used were: (*a*) electively resected primary CRC; (*b*) processing of fresh paired normal mucosa-tumor samples within 30 min after tumor removal; (*c*) available DNA from normal and tumor tissue for biomolecular analyses; and (*d*) access to accurate follow-up information. Briefly, the following exclusion criteria were used: (*a*) history of previous neoplasias; (*b*) patients from families with familial adenomatous polyposis or hereditary nonpolyposis CRC with a highly penetrant genetic predisposition to CRC; (*c*) synchronous or metachronous CRC; and (*d*) chemotherapy or radiation therapy before surgery. A resection of the primary CRC was performed in all of the cases. A total of 137 patients was potentially cured by radical surgical tumor resection with regional en bloc lymphadenectomy proximally up to the origin of the vascular trunks. Twenty-three patients had either nonradical surgery or distant metastases. To avoid evaluator variability in the patients, all of the resection specimens and microscopic slides were meticulously examined by two independent pathologists (R. M. T. and M. M.), who were not aware of the original diagnosis and of the results of the molecular analyses. The complete excision of the primary tumor was proven by the histopathological examination on 3–5 consecutive 4 μm-thick sections of the proximal and distal resected margins, respectively. All of the tumors were histologically confirmed to be CRCs. In addition, the pathologists assessed tumor site (proximal or distal tumors), tumor size, pathological stage, tumor grade (histological differentiation), presence or absence of lymph node metastases, tumor growth (expansive or infiltrative), tumor type (adenocarcinoma NOS or mucinous), presence or absence of vascular and lymphatic invasion or tumor lymphocytic infiltrate. According to Turnbull's modification of Dukes' system (16) the tumors were staged from A to D. Mucinous carcinomas were defined by the presence of mucin in $>$50% of the tumoral area as determined from the available histological sections. Finally, metastatic cases were identified by clinical and histopathological analyses of neoplastic cells in organs such as the lymph nodes, the liver, and so on, along with the primary tumor. Patients with Dukes' stage A and B CRC were treated with surgery alone, whereas only 10 patients with Dukes' stage C received adjuvant chemotherapy with 5-fluorouracil, leucovorin, and levamisole, because in the pre-1991 period almost none of the patients received adjuvant treatment. Patients with nonradical surgery and/or distant metastases were treated by 5-fluorouracil and leucovorin. Postoperatively, all of the patients were checked at 3-monthly intervals for the first 2 years, at 6-monthly intervals for the next 2 years, and annually thereafter. The follow-up program included a clinical examination, blood tests (including carcinoembryonic antigen assay), annual chest radiography, and endoscopy. Abdominopelvic computed tomography scan was also performed each year for the first 2 years. Disease relapse (local recurrence or distant

Table 1 Patient characteristics (n = 160)

	No. patients
Sex	
Male	76
Female	84
Age	
<55	29
55–75	96
>75	35
Site	
Proximal tumor	31
Distal tumor	129
Tumor size (cm)	
≤5	60
>5	100
Dukes' stage	
A	40
B	51
C	41
D	28
Node status	
Negative	101
Positive	59
Tumor growth	
Expansive	20
Infiltrative	140
Tumor grade	
Well differentiated (G1)	23
Moderately differentiated (G2)	104
Poorly differentiated (G3)	33
Tumor type	
Adenocarcinoma NOS	137
Mucinous	23
Lymphoemative invasion	
None	45
Present	115
Lymphocitic infiltrate	
Prominent	48
Nonprominent	112
Surgery	
Curative resection	137
Noncurative resection	23

metastases) was confirmed histologically where possible. Written informed consent was obtained from all of the patients included in this study. Information on survival (DFS and OS) was obtained directly from clinical charts and through the Oncology Section at our Institution. Clinicopathological and follow-up data of all of the patients have been recorded prospectively in a computerized registry database (Table 1).

Tissue Handling and DNA Extraction. Multiple samples (6–10) of the primary tumor tissue were taken from different tumor areas (including the core and the invasive edge of the tumor). The portion of primary tumor was obtained by superficial biopsy of either the tumor bulk or the edge of the malignant ulcer for more infiltrative cancer. All of the tissues were carefully trimmed to remove as much non-neoplastic tissue as possible, avoiding the nonviable areas. Furthermore, multiple samples of normal mucosa (as confirmed by histology) were taken from a macroscopically uninvolved area 20–40 cm away from the tumor site, to be used as control for biomolecular and flow-cytometric analysis. The tissues were bisected, one-half of each sample was processed for pathological examination, and the remaining half of the sample pool was immediately frozen and stored at −80°C until analyzed. The adequacy of the material was checked on frozen tissue sections, and only tissue samples with >80% tumor content were used in subsequent biomolecular and flow-cytometric analysis. Where present, areas with a high content of non-neoplastic cells were removed from the frozen block with a scalpel. Evaluation of each biomolecular variable (p53 alterations, DNA-ploidy, and SPF) was performed independently by researchers who had no knowledge of the clinical data for the samples. High molecular weight genomic DNA was extracted as described previously (17) from primary CRC and normal colon specimens.

Detection of *p53* Gene Mutations. Mutations within the *p53* gene were detected by SSCP analysis following PCR amplification of the exons 5–8, performed as described previously (18). In every instance, negative (DNA was replaced with water) controls were amplified by PCR and included in the experiment. In all of the 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. One-hundred ng aliquots of the amplified DNA fragments, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA) were denatured and analyzed by SSCP analysis. PCR-SSCP analysis was repeated twice for each sample to minimize the possibility of artifacts because of contamination or polymerase errors, and interpretation of SSCP analysis was performed by consensus of two investigators. DNA of normal 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 germ-line mutations or polymorphisms. Individual single-strand DNA fragments with shifted mobilities, compared with normal control, were electroeluted from polyacrylamide gel, reamplified, and sequenced as described previously (19).

Flow-Cytometric Analysis. DNA flow cytometric examination was performed on samples of frozen healthy mucosa and tumor tissue as described previously (20, 21). Briefly, after being thawed a room temperature, the samples were mechanically disaggregated. The suspension was subsequently diluted to a concentration of $\sim 2 \times 10^6$ nuclei/ml. One aliquot of the suspension was used for the preparation of standard reference slides stained with Papanicolaou stain to confirm the presence of tumoral nuclei. For the DNA analysis, the nuclei were stained in a solution containing 50 μg/ml propidium iodide (Calbiochem, La Jolla, CA), 50 mg/ml RNase A (Sigma Chemical Co., St. Louis, MO), and 0.03% NP40 (Sigma) for 30 min at room temperature in the dark. Before staining, a cellular suspension obtained from healthy mucosa was added to each sample as a internal standard reference. A FACSort flow cytometer (Becton Dickinson, CA) was used to obtain data. The suspension of tumoral nuclei (ranging from 20,000 to 65,000) and those used as standard reference were measured both separately and after mixing. Double or triple DNA measurements were performed on all of the samples.

DNA ploidy status was expressed as a DI (22). Tumors with a DI of 1 were defined as DNA diploid, and tumors with lower or higher DI values were considered DNA aneuploid if they contained >10% aneuploid cells. The DNA-aneuploid results were additionally subdivided into monoclonal (with only one DNA-aneuploid peak) and multiclonal (with two or more DNA-aneuploid peaks). Proliferative activity was expressed as the fraction of cells in the S phase of the cell cycle. SPF was calculated according to a program using the mathematical method of Dean and Jett with Multicycle software

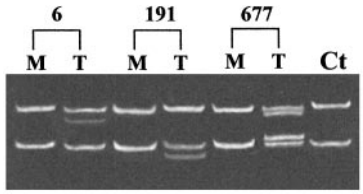

Fig. 2. SSCP analyses of exon 6 of the *p53* gene, amplified from CRC and mucosa genomic DNA of 3 patients. In each *pair of lanes* the normal tissue DNA is at the *left* and the tumor DNA is at the *right*. The *extra bands* visualized in lines 2, 4, and 6 correspond to single-strand DNA molecules harboring mutations in codon 195 (ATC to TTC), 192 (CAG to TAG), and 206/207 (TTG to TT- and GAT to -AT, respectively) as confirmed by sequencing. *Lane 7* shows negative control (*Ct*) DNA wild-type.

(Phoenix Flow Systems, San Diego, CA), including systematic background subtraction (23).

Statistical Analysis. Fisher's exact test (StatXact Turbo; Cytel Software Corporation, Cambridge, MA) was used to evaluate the associations between biological variables. The relationship of different prognostic variables to DFS and OS was assessed univariately by the Kaplan and Meier method. 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 CRC and those surviving. DFS was measured from the day of primary surgery to the date of the first relapse (locoregional or metastatic). Significant differences among survival curves were checked by the log-rank test and Wilcoxon test, or a test for trend when appropriate. Multivariate analysis was carried out by Cox proportional hazards model, using a backward procedure (24). *P*s < 0.05 were considered significant.

Results

Mutation Analysis of *p53* Gene. Mutation analysis of exons 5–8 of the *p53* gene was performed on genomic DNA from primary CRCs of 160 patients by the PCR-SSCP technique. The absence of abnormal bands was assessed in at least two independent PCR-SSCP analyses for each exon. Aberrantly migrating bands were found in 42.5% (68 of 160) of the cases (Fig. 2). Sequence analysis of the DNA fragments with altered electrophoretic mobility made it possible to establish the exact site and nature of the genetic alteration in 65 tumor samples. Overall, 84 p53 mutations were identified in 68 of the 160 screened CRCs, and sequence data were obtained for 81. The features of the p53 mutations are summarized in Table 2. Of the 84 mutations, 19% (16 of 84) were in exon 5, 32% (27 of 84) in exon 6, 36% (30 of 84) in exon 7, and 13% (11 of 84) in exon 8. Fourteen tumor samples were found to harbor two (9 in the same exon and 3 in two different exons) or three (2 in two different exons) different p53 mutations. Nineteen of the 81 sequenced mutations (23%) were found to be frameshifts (15 microdeletions and 4 microinsertions), whereas 62 (77%) were single-nucleotide substitutions. Eighty-two percent (51 of 62) of the latter were missense (45 of 62) or nonsense (6 of 62) mutations, whereas silent mutations were found in 11 cases (9 of them being in codon 213, a site of polymorphism identified previously). Moreover, transitions (81%, 50 of 62) were far more frequent than transversions (19%), G:C to A:T mutations (50% at CpG sites) being the most frequently represented (76%). Forty-two different mutations spanned 32 codons, whereas 30 mutations (37%) clustered in 4 codons: 9 cases each in codons 248 and 249, 7 cases in codon 244, and 5 cases in codon 273. No germ-line mutations were found indicating that in every case the change was somatic. Fifty-seven percent of the mutations (48 of 84) occurred in highly conserved domains (areas II-V). Accordingly, tumors with p53 mutations were classified in two groups: 56% of the cases (38 of 68 cases) with mutations in conserved areas of the *p53* gene (conserved) and 44% (30 of 68 cases) with mutations outside the conserved areas (nonconserved). In addition, by taking into account the specific functional and structural domains of p53 affected by the mutations, the cases were also classified as follows: 18 of 68 cases (26%) with mutations of the L3 loop, 11 of 68 cases (16%) with mutations of the LSH motif, and 39 of 68 cases (58%) with mutations outside L3 loop and LSH. Because silent mutations do not determine any amino acid change in the protein, they have been included in the wild-type group for statistical analysis.

Cellular DNA Content Evaluation. Adequate DNA histograms were obtained for all of the normal and tumoral tissues by flow cytometry. The coefficients of variation of the DNA-diploid peak ranged from 2.5 to 4.8% (median 3.4%). DNA aneuploidy was found in 75% of the cases (120 of 160), whereas 18% of these (22 of 120) showed multiclonality. Fig. 3 shows the distribution of the DI values in the 120 DNA-aneuploid cases.

SPF Evaluation. The SPF ranged from 2.1 to 32.6% (median: 18.3% and interquartile range: 14.1–21.7%). By using the SPF median value as a cutoff point, tumors were accordingly divided into low ($\leq$18.3%) and high ($>$18.3%) SPF tumors.

Relationship between Biomolecular Indicators and Clinical Data. p53 mutations in the conserved regions were more frequent in distal cancers ($P < 0.05$), in DNA aneuploid ($P < 0.01$), and in high SPF tumors ($P < 0.01$; Table 3). Moreover, p53 mutations affecting L3 loop were significantly associated with high SPF ($P < 0.05$; Table 4). No significant relationship was seen between the presence (any mutations) or type of p53 mutations (missense *versus* frameshift and transitions *versus* transversions *versus* frameshift) and the clinicopathological variables analyzed. DNA aneuploidy was associated with distal tumors ($P < 0.01$), histological grade (G3; $P < 0.05$), advanced Dukes' stage (C and D; $P < 0.01$), lymph node metastases ($P < 0.01$), and high SPF. The median SPF of DNA-aneuploid tumors was 19.2%, whereas that of the DNA-diploid tumors was 12.4% ($P < 0.01$; Table 4).

Uni- and Multivariate Analysis of Prognostic Factors. The median follow-up time in our study group was 71 months (range 34–115 months). The median survival of the whole group was 43 months. At univariate analysis, distal cancers, advanced Dukes' stage, node status positive, lymphohematic invasion, DNA aneuploidy, and high SPF, the presence, type (frameshift), or site (in conserved regions, affecting L3) of p53 mutations proved to be significantly related to quicker relapse, whereas these same factors, and in addition infiltrative tumor growth, prominent lymphocytic infiltration, and noncurative resection, were significantly related to shorter OS (Table 5). Fig. 4 shows the probability of DFS (Fig. 4*a*) and OS (Fig. 4*b*) according to p53 mutations in specific structural domains. The significant variables at univariate analysis were entered in a Cox proportional hazards model with backward elimination. The major significant predictors for both disease relapse and death were advanced Dukes' stage, aneuploid tumors, high SPF, and p53 mutations affecting L3 loop, whereas lymphohematic invasion was the only independent factor for relapse and noncurative resection for death (Table 6).

Table 2 Localization and type of mutations in 68 patients with CRC

Sample	Mutated exon	Mutated codon	Nucleotide change	Amino acid change	Type mutation	CD	Zn-BD
3 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
6 K	6	195	atc→ttc	ile→phe	Missense	N	L2
13 K	8	273	cgt→cat	arg→his	Missense	Y	LSH
19 K	5	158	cgc→cac	arg→his	Missense	N	
26 K	7	238+	tgt→tat	cys→tyr	Missense	Y	L3
		249	agg→ag-	arg→	Frameshift	Y	
27 K	5	155	acc→ac-	thr→	Frameshift	N	
28 K	5	174	agg→aag	arg→lys	Missense	Y	L2
30 K	5	141	tgc→tac	cys→tyr	Missense	Y	
35 K	5	151	ccc→tcc	pro→ser	Missense	N	
36 K	5	158	cgc→cac	arg→his	Missense	N	
63 K	6	204+	gag→tag	glu→STOP	Nonsense	N	
		206	ttg→tag	leu→STOP	Nonsense	N	
70 K	7	244+	ggc→agc	gly→ser	Missense	Y	L3
		249	agg→ag-	arg→	Frameshift	Y	
77 K	8	272	gtg→atg	val→met	Missense	Y	LSH
102 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
110 K	7	244+	ggc→agc	gly→ser	Missense	Y	L3
		249	agg→ag-	arg→	Frameshift	Y	
114 K	5+	141	tgc→cgc	cys→arg	Missense	Y	
	6	204	gag→tag	glu→STOP	Nonsense	N	
125 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
130 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
147 K	8	282	cgg→tgg	arg→trp	Missense	Y	LSH
172 K	5	158	not sequenced			N	
191 K	6	192	cag→tag	gln→STOP	Nonsense	N	L2
195 K	7	244+	ggc→agc	gly→ser	Missense	Y	L3
		249	agg→ag-	arg→	Frameshift	Y	
207 K	6	213	cga→cgg	arg→arg	Silent	N	
221 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
234 K	5	152	ccg→cct	pro→pro	Silent	N	
237 K	6	196	cga→ctga	arg→	Frameshift	N	
245 K	6	220	tat→tgt	tyr→cys	Missense	N	
254 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
333 K	7	255/	atc/aca→	ile/thr→	Frameshift	Y	
		/256	at-/–a	ile/-			
334 K	7	253+	acc→ac-	thr→	Frameshift	Y	
		255	atc→a-c	ile→	Frameshift	Y	
338 K	7	244+	ggc→agc	gly→ser	Missense	Y	L3
		249	agg→ag-	arg→	Frameshift	Y	
344 K	6	201	ttg→ttc	leu→phe	Missense	N	
351 K	5	152	ccg→cccc	pro→	Frameshift	N	
368 K	6	202	cgt→cgct	arg→	Frameshift	N	
371 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
398 K	6+	220	tat→tgt	tyr→cys	Missense	N	L3
	7	244+	ggc→agc	gly→ser	Missense	Y	
		249	agg→ag-	arg→	Frameshift	Y	
406 K	5	174	agg→aag	arg→lys	Missense	Y	L2
416 K	6+	213	cga→cgg	arg→arg	Silent	N	L3
	7	248+	cgg→cag	arg→gln	Missense	Y	
		249	agg→ag-	arg→	Frameshift	Y	
421 K	5	177	ccc→cc-	pro→	Frameshift	Y	L2
424 K	6	213	cga→tga	arg→STOP	Nonsense	N	
427 K	5	141	tgc→tac	cys→tyr	Missense	Y	
449 K	6	213	cga→cgg	arg→arg	Silent	N	
452 K	7	248	cgg→tgg	arg→trp	Missense	Y	L3
463 K	8	273	cgt→cat	arg→his	Missense	Y	LSH
466 K	6	214	cat→caat	his→	Frameshift	N	
468 K	6+	213	cga→cgg	arg→arg	Silent	N	
	8	282	cgg→tgg	arg→trp	Missense	Y	LSH
499 K	8	278	cct→tct	pro→ser	Missense	Y	LSH
508 K	6+	193	cat→cag	his→gln	Missense	N	L3
	7	242	tgc→tac	cys→tyr	Missense	Y	
543 K	8	272	gtg→atg	val→met	Missense	Y	LSH
552 K	8	273	cgt→cat	arg→his	Missense	Y	LSH
558 K	6	213	cga→tga	arg→STOP	Nonsense	N	
569 K	6	213	cga→cgg	arg→arg	Silent	N	
620 K	6	213	cga→cgg	arg→arg	Silent	N	
627 K	5	151	ccc→acc	pro→thr	Missense	N	
629 K	6	not sequenced				N	
638 K	6	213	cga→cgg	arg→arg	Silent	N	
645 K	8	273	cgt→cat	arg→his	Missense	Y	LSH
677 K	6	206/	ttg/→tt-/leu/→		Frameshift	N	
		/207	/gat/-at/asp				
678 K	7	244+	ggc→gcc	gly→ala	Missense	Y	L3
		249	agg→cgg	arg→arg	Silent	Y	
686 K	6	206/	ttg/→tt-/	leu/→	Frameshift	N	
		/207	/gat/-at/asp				
700 K	6	213	cga→cgg	arg→arg	Silent	N	
713 K	8	278	cct→tct	pro→ser	Missense	Y	LSH
706 K	5	163	tac→ttc	tyr→phe	Missense	N	L2
747 K	6	213	cga→cgg	arg→arg	Silent	N	
757 K	5	141	tgc→tcc	cys→ser	Missense	Y	
789 K	8	273	cgt→cat	arg→his	Missense	Y	LSH
817 K	7	244+	ggc→agc	gly→ser	Missense	Y	L3
		249	agg→ag-	arg→	Frameshift	Y	
861 K	6	not sequenced				N	

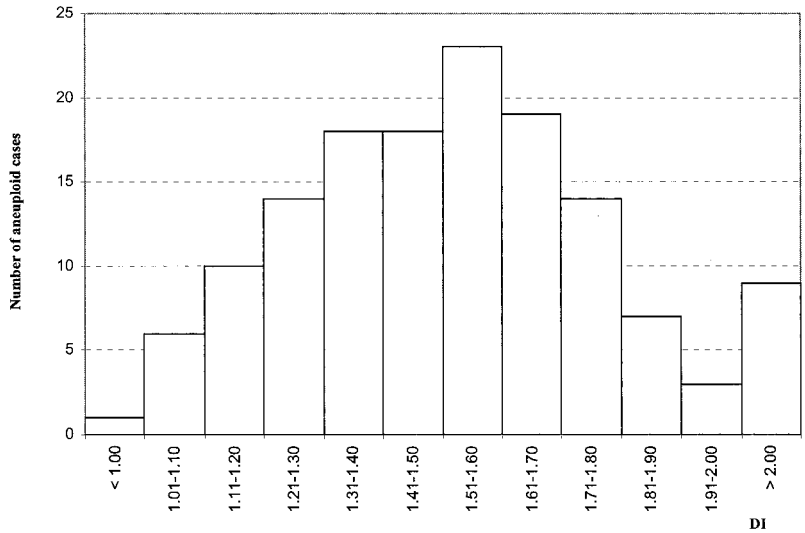

Fig. 3. Distribution of the DI values in 120 DNA-aneuploid cases (98 monoclonal and 22 multiclonal).

Table 3 Relationships of p53 mutations in the highly conserved domains or in the functional/structural domains, and clinicopathological and biological variables of the 160 CRC patients

	p53							
	No mutation (%)	Nonconserved (%)	Conserved (%)	*P*	L3 (%)	LSH (%)	Outside L3/ LSH (%)	*P*
Total	100 (62)	22 (14)	38 (24)					
Site								
Proximal tumors	26 (84)	3 (10)	2 (6)					
Distal tumors	74 (57)	19 (15)	36 (28)	<0.05				
DNA ploidy								
Diploid	35 (88)	2 (5)	3 (7)					
Aneuploid	65 (54)	20 (17)	35 (29)	<0.01				
SPF								
≤18.3	60 (74)	10 (12)	11 (14)		4 (5)	5 (6)	12 (15)	
>18.3	40 (51)	12 (15)	27 (34)	<0.01	14 (18)	6 (8)	17 (22)	<0.05

Discussion

Previous analyses performed by several researchers on different types of tumors has shown that most of the p53 mutations (~95%) affect exons 5–8, which code for residues 130–286, the most important region for the folding and, therefore, for the stabilization of the tertiary structure of the protein (core domain), and which contains the site-specific, DNA-binding domain (9).

In our screen for mutations in exons 5–8 of p53 on genomic DNA from primary CRCs of 160 patients, we observed a mutation frequency of 42% (68 of 160), within the fairly wide range of values reported previously in CRC (23–61%) (10, 11, 25–28). This variability can be explained by several factors, such as the different methods used to assess p53 mutations (SSCP, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, and direct sequencing), the type of tumor storage (fresh/frozen tissue and paraffin-embedded blocks), an intrinsic tumoral heterogeneity, and, in addition, more specific features of the patient cohorts entered in the study, in particular, histopathologic staging and grading of the tumor. In fact, patients at an advanced Dukes' stage (C and D) and/or with poorly differentiated tumors (G3) generally present a higher rate of p53 mutations (28, 29). In accordance with several other reports (10, 26, 28), 57% of all of the mutations observed in our series (48 of 84) were in four of the five highly conserved areas of the gene, which include two important regions for p53 binding to DNA. One of these contains the amino acids needed for DNA interaction, in particular those that are part of the L3 Zn-BD and of the LSH motif. In our own series, 21% (18 of 84) of the mutations occurred in L3 and 13% (11 of 84) in LSH, in accordance with the results reported by Borresen-Dale *et al.* (12). Our data confirm that arginines 248 and 273, amino acids of these domains interacting directly with DNA, are among the most frequently mutated residues (in our series: 9% and 5%, respectively). On the contrary the absence of codon 175 mutations in our series suggests that mutations at specific codons may be an indication of specific exposures to toxic agents or of genomic susceptibility. The second region, which, when mutated, is also responsible for the loss of p53 DNA-binding capacity includes the amino acids localized in L2, and needed for the folding and stabilization of the central domain (9). Mutations in this area were observed less frequently in our own series (7%).

Flow-cytometric analysis of CRCs shows DNA aneuploidy rates ranging from 39 to 89% (4, 30). The aneuploidy rate (75%) observed in our study is among the highest thus far reported in the literature, probably for two main reasons: (*a*) the multiple sampling performed in all of the cases studied, which

Table 4 Relationships of DNA ploidy to clinicopathological and biological variables of CRCs

	DNA ploidy				*P*
	Diploid		Aneuploid		
	n	%	*n*	%	
Total	40	25	120	75	
Site					
Proximal tumors	19	61	12	39	
Distal tumors	21	16	108	84	<0.01
Tumor grade					
G1	12	48	13	52	
G2	21	21	81	79	<0.05
G3	7	21	26	79	
Dukes' stage					
A + B	30	33	61	67	
C + D	10	14	59	86	<0.01
Node status					
Negative	32	32	67	68	
Positive	8	13	53	87	<0.01
SPF					
≤18.3%	32	40	47	60	
>18.3%	8	10	73	90	<0.01

Table 5 Kaplan-Meier DFS (n = 138) and OS (n = 160) analysis of biological variables in patients with CRC

	No. patients	DFS (%) 5 yrs	*P*	No. patients	OS (%) 5 yrs	*P*
DNA-ploidy status[a]						
Diploid	38	75		40	77	
An. Monoclonal	81	34		98	33	
An. Multiclonal	19	10	<0.01	22	9	<0.01
SPF[b]						
≤18.3%	72	60		81	59	
>18.3%	66	22	<0.01	79	21	<0.01
p53[c]						
No mutations	88	51		100	49	
Any mutations	48	25	<0.01	58	26	<0.01
No mutations	88	51		100	49	
Mutation in nonconserved areas	19	32		20	40	
Mutation in conserved areas	29	21	<0.05	38	18	<0.05
No mutations	88	51		100	49	
Mutation outside L3/LSH	26	27		29	34	
Mutations in LSH	9	33		11	27	
Mutations in L3	13	15	<0.05	18	11	<0.01
No mutations	88	51		100	49	
Missense mutations	33	24		39	28	
Frameshift mutations	14	29	<0.05	18	22	<0.05

[a] All DNA-aneuploid subgroups are compared with patients with DNA-diploid tumors.
[b] All high SPF subgroups are compared with patients with low SPF tumors.
[c] All mutation subgroups are compared with patients with no mutations (wild-type p53).

considerably reduces the probably of missing aneuploid clones at analysis; in fact, CRCs are heterogeneous, both from the histopathologic point of view and also with regard to cellular DNA content (31, 32); and (*b*) the choice of freezing to −80°C rather than paraffin-embedding as sample storage method. Although the analysis of paraffin-embedded samples permits the retrospective evaluation of many cases with suitable follow-up, this type of storage may compromise the reliability of the results, because of the presence of a relatively large quantity of debris, to poor histogram resolution and to high coefficients of variation.

Our results show a significant relationship between p53 mutations in the conserved regions and flow-cytometry parameters, and also an association between DNA aneuploidy and SPF. The association between p53 mutations in the conserved regions, and aneuploidy and high SPF may be related to the well-known role played by p53 as a brake to the cell cycle and as guardian of the integrity of the genome. Interestingly, we additionally found that p53 mutations in the L3 domain are associated only with high SPF and not with aneuploidy. This might be taken as an indication that the pathways through which p53 restrains cell proliferation and prevents mitosis in cells with aberrant mitotic spindles are activated independently by p53, and that its function as a transcriptional regulator is differentially affected by mutations in L3 loop *versus* mutations in other adjacent conserved regions. In the first case, only a subset of the genes regulated by p53 would no longer be under its control, whereas in the second, p53 would also lose its capacity to interact with additional promoters, and thus activate/repress the transcription of a larger number of (or even all) its target genes. Should this interpretation of our data be confirmed by additional studies, this may be the first evidence of a direct correlation between specific p53 mutations and loss of distinct functional properties of p53 *in vivo*. The association between DNA-aneuploidy and high SPF found in our study might result from higher proliferative activity of the aneuploid clones or else from a prolongation of S phase in an altered cell cycle with a higher risk of additional genetic alterations and higher probability of development of populations containing aneuploid DNA. In accordance with Jernvall *et al.* (33), we observed a higher incidence of mutations in the conserved areas of p53 in distal CRC, as well as a significant association between DNA ploidy and the primary tumor site: as in most of the reports in literature (34–36), we too found a higher rate of aneuploidy in distal CRC and higher diploidy in proximal CRCs. These data, together with the observation that microsatellite instability is found mainly in diploid tumors and in those of the proximal colon (37), support the hypothesis of Beart *et al.* (38), that carcinomas of the proximal and distal large intestine may well be "biologically different diseases" reflecting distinct carcinogenic pathways.

In our prospective study, based on univariate and multivariate analyses with established prognostic indicators (such as Dukes' stage, tumor grade, and lymphohematic invasion), we have found that DNA ploidy, SPF, and p53 mutations affecting the L3 region, but not any mutation or other specific subgroups of mutations in p53, are significant and independent prognostic factors for DFS and OS in patients with CRC who have undergone surgical resection. This finding confirms and extends recent reports on the prognostic significance of p53 mutations, which is still a matter of debate in the literature. In fact, whereas in several studies in which any p53 mutations identified in the tumoral cells were taken into account, they were found to be a significant prognostic factor (10, 11, 27), in others they did not appear to have any relation with clinical behavior (26, 29, 39). However, those studies in which mutations in specific domains of p53, rather than any mutation, were considered, provide promising results, indicating that not all of the mutations affect p53 biological properties in the same way and suggesting that specific mutations in regions that are important for protein function have a more significant prognostic impact than any p53 mutations (28). Nevertheless, even studies involving mutations in highly conserved p53 areas have not provided definitive evidence on this point. In fact the prospective study

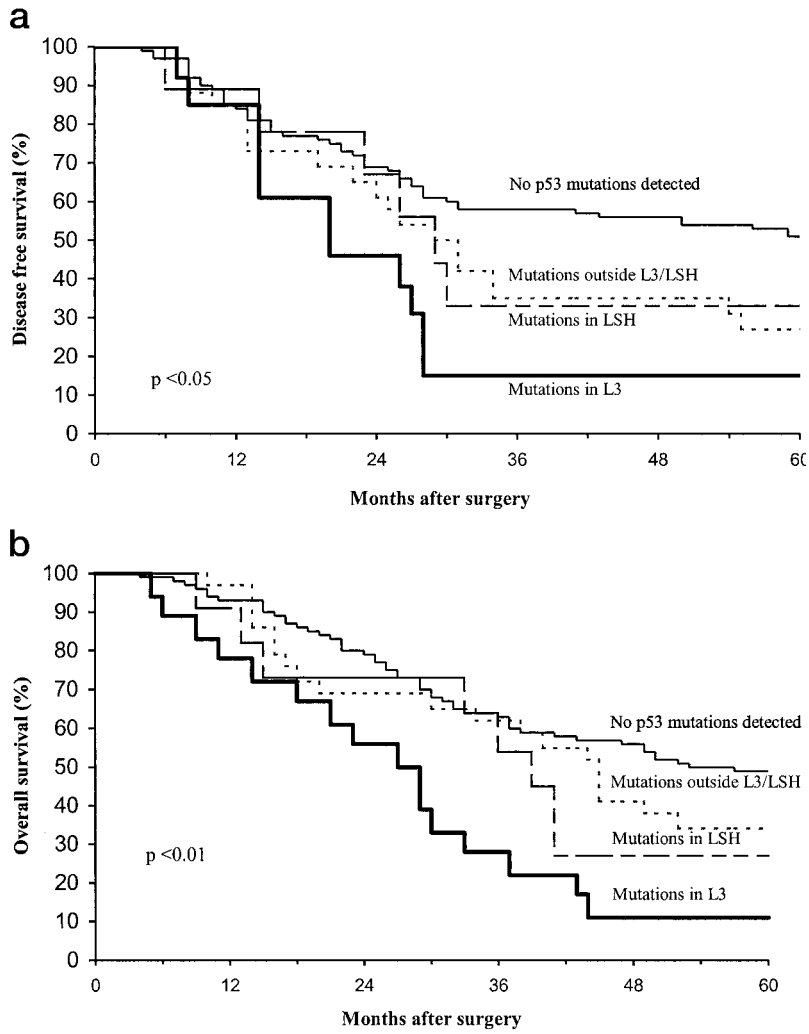

Fig. 4. DFS (*a*) and OS (*b*) of 160 patients with CRC according to p53 functional and structural domains.

Table 6 Cox proportional hazards analysis to predict the HR of relapse or death in CRC patients

	Relapse (n = 138)		Death (n = 160)	
	HR (95% confidence interval)	*P*	HR (95% confidence interval)	*P*
Dukes' stage				
D *vs.* A	3.20 (1.32–7.71)	<0.01	7.29 (3.08–17.2)	<0.01
Surgery				
Noncurative resection *vs.* curative resection			3.85 (1.74–8.49)	<0.01
DNA ploidy				
An. Monoclonal *vs.* Diploid	2.63 (1.25–5.49)	<0.01	2.26 (1.12–4.59)	<0.05
An. Multiclonal *vs.* Diploid	6.40 (2.75–14.9)	<0.01	5.04 (2.25–11.3)	<0.01
p53				
L3 *vs.* no mutation	2.14 (1.06–4.32)	<0.05	2.29 (1.24–4.25)	<0.01
SPF				
>18.3% *vs.* ≤18.3%	2.64 (1.62–4.31)	<0.01	2.23 (1.40–3.54)	<0.01
Lymphohematic invasion				
Present *vs.* none	2.26 (1.25–4.11)	<0.01		

conducted by Goh *et al.* (28) on 192 CRCs has shown that tumors with such mutations were more aggressive than those with mutations in other areas; on the contrary, Kressner *et al.* (10) found in 189 CRCs that mutations occurring outside conserved regions were associated with a more unfavorable prognosis. A clearer picture results from studies regarding mutations within the L2, L3, and LSH domains; analyses of tumors in different regions of the body (head and neck, esophagus, breast,

lung, and ovary) have demonstrated that mutations in these areas have a strong clinical impact (15, 40–44). More specifically, in the only study published thus far on CRCs (12), based on univariate analysis of 222 cases, mutations involving L3 were associated with shorter survival rate. As Skaug *et al.* (15) has suggested, because L2, L3, and LSH domains mutations destabilize the DNA protein-binding domains more than any other type of mutation, they might alter more drastically the biological function of p53 and might thus contribute to the development of clinically more aggressive tumors; they are probably also more resistant to chemotherapy (14). The data resulting from the present study would confirm the tendency of these reports. We found, in fact, that p53 mutations in the L3 domain were significantly associated with risk of disease relapse and of death in CRCs, and can be considered as independent prognostic variables. Whether cell DNA content is a significant prognostic factor in CRC is still not clear from the data reported in literature (4). Whereas several research groups have suggested that DNA ploidy is an independent variable (36, 45, 46), others have reported that this biological variable is not associated with clinical outcome in CRC (47–49). These conflicting results may be partly because of several factors, such as patient selection, number of cases studied, intratumoral heterogeneity, sampling methods, analytic techniques, lack of standardization and inadequate control of the techniques from one laboratory to another, and interpretation of results. From the clinical point of view, CRC containing multiple abnormal stemlines ("DNA-multiploid tumors") might have a more adverse prognosis than those containing a single abnormal stemline (2, 3). In our own study, in fact, Cox proportional hazards analysis of the patient subgroup with multiclonal tumors showed both a higher risk of disease relapse and of death.

The prognostic value in CRC of SPF, despite the use of different mathematical models, seems to be clearer. Literature reports almost all agree that this biological variable is a major determinant of biological aggressiveness and has a predictive role in clinical outcome (5, 39). This is in accordance with our results in which SPF was identified as independent prognostic factor.

In conclusion, if our results on these biological indicators (specific p53 mutations, DNA ploidy, and SPF) are confirmed by large prospective studies, it will be possible to provide additional and important useful information for prognosis and a rational basis for the development of more specific clinical treatments (more radical surgery, and/or adjuvant chemotherapy or radiotherapy). In particular, efforts should therefore be directed toward subsets of CRC patients with a high risk of relapse or death, especially those with Dukes' stages B (Astler-Coller B2) and C. Furthermore, biomolecular variables of predictive relevance combined with pathological staging might be considered as stratification factors in studies containing multiple treatment approaches to identify biological indicators of response to therapy.

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