

Lack of p53 Nuclear Immunostaining Is Not Indicative of Absence of TP53 Gene Mutations in Colorectal Adenocarcinomas

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Multiple studies using primary tumors have reported that alterations in p53 expression and detection of TP53 mutations are associated with clinical aggressiveness and poor response to specific therapies. However, there is no general agreement regarding the optimal technical approach to the analysis of p53. We have studied a series of 100 primary colorectal adenocarcinomas by immunohistochemistry with the monoclonal antibody PAb1801, and single-stranded conformation polymorphism (PCR-SSCP, exons 4–8) followed by direct sequencing of shifted bands. p53 Nuclear staining was undetectable (score 0) in 29 of 100 cases. However, gene mutations were detected in 15 of these cases, with all of these mutations leading to abnormal proteins. p53 Nuclear staining was detectable and scored as less than 10% tumor cells positive in 15 of 100 cases but was still considered to be displaying a p53-negative phenotype because the cut-off value for positivity was 10% positive tumor cells. Nevertheless, TP53 gene mutations were detected in 2 of these cases. p53 Nuclear immunoreactivities were detectable and scored as more than 10% tumor cells positive in 56 cases, considered the p53-positive phenotype. TP53 gene mutations were identified in 51 of these 56 cases. These results reveal that immunohistochemical assessment does not predict TP53 mutation status in colorectal adenocarcinoma, mainly in cases displaying absence of nuclear staining. It is thus concluded that molecular profiling should be conducted in parallel with immunophenotyping when analyzing colorectal tumors for p53 status.

Key Words: colorectal carcinoma, p53, immunohistochemistry, sequencing, 17p allelic losses

Applied Immunohistochemistry & Molecular Morphology 11(2):130–137, 2003.

Mutations of the TP53 tumor suppressor gene and altered patterns of p53 expression are among the most frequent abnormalities reported in human cancer.[1–4] p53 functions as a cell cycle checkpoint, sensing DNA damage and modulating a reversible G1 arrest that allows DNA repair to occur; if optimal repair is not achieved, it activates an apoptotic signal, aborting the cell cycle.[4]

Manuscript received September 27, 2002; accepted October 29, 2002.

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Although pathology stage at the time of surgery is presently the main prognostic factor for patients with colorectal cancer;[5–7] molecular alterations acquired during tumor progression appear to offer additional predictive information.[8–11] Moreover, because p53 function is required for an efficient activation of apoptosis, its alteration has been related to lack of response to certain therapies.[12]

The most common mechanism for loss of p53 function is acquisition of a point mutation in 1 allele and deletion of the remaining allele at 17p11.[1,13,14] Loss of the short arm of chromosome 17 and of the TP53 locus have been shown to provide prognostic information in different tumor types, including colorectal cancer.[15–18] In addition to this mechanism, p53 inactivation occurs because of the dominant negative effect of some mutant p53 proteins, because in its final functional state, p53 needs to form tetramers.[19–21] Finally, specific TP53 point mutations have been reported to produce mutant products that acquire gain of function. Such a paradigm for p53 mutations has been associated with enhanced tumorigenicity, metastatic potential, and resistance to certain therapeutic agents.[22–24]

Detection of nuclear overexpression of p53 products by immunohistochemistry (IHC) has been associated with the identification of TP53 gene mutations in a large number of clinical studies.[25,26] Although the half-life of the wild-type p53 protein is short, usually less than 30 minutes, some amino acid substitutions resulting from missense mutations are known to produce stabilization of the product and prolongation of its half-life.[27,28] This accumulation allows microanatomic immunolocalization in tissue sections from primary tumor material. Despite general acceptance of this technical approach as a reflection of an underlying mutation,[29–31] the absence of nuclear immunoreactivity does not rule out alteration of the TP53 gene. In fact, nonsense mutations may produce either unstable transcripts or truncated proteins that lack the nuclear localization signal, rendering negative staining results.[25] These events may account for approximately 20% of the TP53 mutations detected in particular tumor types.[32,33]

We undertook the current study to define the degree of concordance between different assays aimed at the detection of TP53 mutations and altered patterns of p53 expression in colorectal cancer. This study was also conducted to assess the potential clinical relevance of identifying p53 altered genotypes or phenotypes in colorectal adenocarcinomas.

MATERIALS AND METHODS

Patient Characteristics and Tissues

Between February 1996 and June 1999, 110 tumor samples were collected from 106 patients surgically treated for colorectal cancer in the area of Barcelona, Spain. A cohort of 100 tumors corresponding to 96 patients was included in this study, because the remaining 10 tumors (9% of total specimens) were excluded because of tissue characteristics, low quality of DNA, or deficient amplification from either normal or tumor samples.

Demographic data on this cohort may be summarized as follows: 58 patients were male and 38 female; median age was 66.2 ± 11.6 years (range, 37–93 years). Tumor staging according to the tumor-node-metastasis (UICC) system[34] revealed 2 stage 0, 16 stage 1, 30 stage 2, and 52 stage 3–4. Regional lymph node involvement was as follows: 48 cases had negative nodes (pN = 0), whereas from the remaining 52 cases, 27 had as many as 3 positive nodes (pN = 1), and 25 had more than 3 positive ones (pN = 2). Regarding histologic grading, 39 tumors were classified as low-grade lesions (grade 1, glandular structures in >95% of the tumor), 34 as intermediate-grade (grade 2, 50–95%), and 27 as high-grade (grade 3, <50%). Tumor size was categorized as follows: less than 3 cm (n = 41), 3 to 6 cm (n = 50), and more than 6 cm (n = 9).

Tissues included in the study were fixed in 10% buffered formalin and embedded in paraffin. Representative hematoxylin and eosin-stained sections of tumor, normal mucosa, associated lesions, margins (proximal, distal, and radial), and lymph nodes were examined microscopically in each case to verify pathology stage and tumor grade. This step also allowed for the selection of viable and morphologically representative areas of the specimen from which IHC and DNA extraction were performed.

Antibodies to p53 and Immunohistochemistry

Immunohistochemical analyses were performed using the monoclonal antibody PAb1801 (Ab-2, Oncogene/Calbiochem Laboratories, Boston, MA) as the primary anti-p53 antibody. This antibody recognizes an epitope located between amino acids 32 to 79 of both wild-type and mutant human p53 proteins.[35] The avidin-biotin immunoperoxidase method was performed on 4-μm-thick paraffin-embedded sections. Briefly, sections previously treated with 0.1-M pH6 citrate buffer for antigen retrieval were blocked for 20 minutes with 5% normal horse serum (Jackson, Westgrove, PE), followed by a 2-hour incubation with the appropriately diluted primary antibody (143 ng/mL). After extensive washing, slides were incubated for 30 minutes with biotinylated horse antimouse IgG antibodies (Vector Laboratories, Burlingame, CA) at 1:100 dilution, and then for 30 minutes with avidin-biotin peroxidase complexes (Vector Laboratories), also at 1:100 dilution. Diaminobenzidine (0.05%) was used as the final chromogen and Harris-modified hematoxylin as the nuclear counterstain. Positive controls, which were cases known to show positive nuclear staining, and negative controls, in which the primary antibody was omitted, were included in each experiment.

Immunohistochemical evaluation was performed by at least 2 independent observers who scored the estimated percentage of tumor cells showing nuclear staining. This evaluation and data recording were conducted without previous knowledge of clinicopathologic information or molecular results. p53 Nuclear immunoreactivities were considered positive when at least 10% of tumor cells showed nuclear immunoreactivities.

DNA Extraction and Control Amplification

Eleven serial 5-μm-thick sections were used for each case. One of these sections was used for tissue characterization after hematoxylin and eosin staining. Unstained slides were then aligned by morphology to the stained slide, and corresponding areas were microdissected. Tumor samples were examined microscopically to confirm the specificity of dissection.

Genomic DNA was obtained from the microdissected sections using a proteinase K extraction method, followed by purification with phenol/chloroform and ethanol precipitation, as described previously.[36] To assess the quality of the genomic DNA extracted, 1 μL of a 200 ng/μL dilution was used as template for the amplification of a 268-bp fragment of the human β-globin gene[37] using a GeneAmp Polymerase Chain Reaction (PCR) System 2400 thermal cycler (PE Biosystems, Foster City, CA). Primers used and amplification conditions are summarized in Table 1. Efficiency of the PCR reactions was assessed in 2% agarose gels.

Single-stranded Conformation Polymorphism Analysis and DNA Sequencing for TP53 Mutations

Single-stranded conformation polymorphism (SSCP) analysis was performed according to a slightly modified method previously reported.[38] The primers used were obtained from intron-exon sequences flanking exons 4 to 8 of the human TP53 gene,[39] and their sequences and the amplification conditions are summarized in Table 1. Genomic DNA, 200 ng, was used as template for each amplification with 1.05 U of the Expand High Fidelity PCR System (Boehringer Mannheim Corp, Indianapolis,

TABLE 1. *Primers and conditions used for PCR amplification*

Primers	Sequence	PCR conditions
β-globin gene		
PC04	5′-CAACTTCATCCACGTTCACC-3′	94°C for 5 min; 1 cycle
GH20	5′-GAAGAGCCAAGGACAGGTAC-3′	94°C for 30 sec; 57°C for 30 sec; 72°C for 30 sec; 35 cycles 72°C for 7 min; 1 cycle
TP53 exons 4–8		
P53E4SE	5′-TTCACCCATCTACAGTCCCC-3′	Exons 4, 6 and 7
P53E4AN	5′-TCAGGGCAACTGACCGTGCA-3′	94°C for 5 min; 1 cycle
P53E5SE	5′-TTCCTCTTCCTGCAGTACTC-3′	94°C for 30 sec; 61°C for 30 sec; 72°C for 1 min; 35 cycles
P53E5AN	5′-AGCTGCTCACCATCGCTATC-3′	72°C for 7 min; 1 cycle
P53E6SE	5′-ACAGGGCTGGTTGCCCAGGGT-3′	
P53E6AN	5′-AGTTGCAAACCAGACCTCAG-3′	Exons 5 and 8
P53E7SE	5′-GTGTTGTCTCCTAGGTTGGC-3′	94°C for 5 min; 1 cycle
P53E7AN	5′-GTCAGAGGCAAGCAGAGGCT-3′	94°C for 30 sec; 56°C for 30 sec; 72°C for 1 min; 35 cycles
P53E8SE	5′-TATCCTGAGTAGTGGTAATC-3′	72°C for 7 min; 1 cycle
P53E8AN	5′-AAGTGAATCTGAGGCATAAC-3′	
TP53 locus		
TP53 GT	5′-AGGGATACTATTCAGCCCGAGGTG-3′	94°C for 5 min; 1 cycle
TP53 AC	5′-ACTGCCACTCCTTGCCCCATTC-3′	94°C for 30 sec; 60°C for 30 sec; 72°C for 30 sec; 35 cycles 72°C for 7 min; 1 cycle

IN). After checking in agarose gels, PCR products were denatured and loaded onto nondenaturing polyacrylamide gels (GeneGel Excel 12.5/24, Amersham Pharmacia Biotech, Uppsala, Sweden). As an exception, exon 4 products, because of their large size (308 bp), were digested using the Ava II (New England Biolabs, Hertfordshire, England, UK) restriction enzyme (2.5 U/10 μL reaction) before loading. Electrophoresis assays were run in 2 different conditions (600 V, 25 mA, 15 W, 15°C for 1 hour and 30 minutes; and 400 V, 15 mA, 8 W, 20°C for 2 hours and 30 minutes). A third condition was used for the first 25 cases, but it was finally rejected because no additional information was obtained when comparing results with those obtained using the other 2 conditions. Gels were then silver-stained and finally analyzed by at least 2 different observers.

Samples displaying anomalous mobility patterns were retested to verify the results. Shifted bands were excised from the gel, eluted in water, and used as a template for direct sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK). Both strands were sequenced for each PCR product analyzed.

To assess the accuracy of the SSCP analysis, 10% of the cases not showing mobility changes were randomly selected for both upstream and downstream sequencing of exons 4 to 8.

Detection of Allelic Losses

Amplification of a P53CA dinucleotide repeat[40] was performed to determine loss of heterozygosity (LOH). An aliquot of 200 ng genomic DNA was amplified by PCR using the primers and conditions that are also summarized in Table 1. PCR products previously checked in 2% agarose gels were then analyzed by electrophoresis in the precast polyacrylamide gels described (600 V, 25 mA, 15 W, 15°C for 1 hour and 30 minutes) and subsequently developed by silver staining. Two independent observers conducted visual evaluation of the allelic patterns, and losses were considered when tumor bands either reduced their signal to at least 60% compared with normal bands or were not detected at all.

Statistical Analyses and Methods

The χ^2 test was used to assess statistical association between p53 immunophenotype, TP53 mutations, and TP53 deletion status with demographic and clinicopathologic parameters including age, sex, tumor size, pathologic stage, histologic grade, and lymph nodal involvement. *P* values quoted were 2-tailed and considered indicative of a statistically significant effect when less than or equal to 0.05. The association between IHC, PCR-SSCP sequencing and 17p allelic loss data, and the possible differences in the distribution of groups between significantly related variables, were compared by Fisher exact test. *P* values quoted were 2-tailed and statistically significant when less than or equal to 0.05. The adjustment by the Bonferroni method for multiple testing was made as appropriate, α-values diminishing less than or equal to 0.016.

Both the receiver operating characteristic (ROC) curve and the multivariate logistic-regression analysis were used to evaluate the validity of IHC in predicting TP53 mutations as measured by PCR-SSCP and sequencing.[41,42] ROC analysis plots sensitivity versus specificity in a graphical display, allowing assessment of the validity in a fair manner without the arbitrariness inherent in individual sensitivity-specificity pairs.[42]

Statistical procedures were performed with SPSS 8.0.1 (Statistical Package for the Social Sciences Inc, Chicago, IL) and Statistical Graphics Software Plus 4.0 (Manugistics Inc, Rockville, MD).

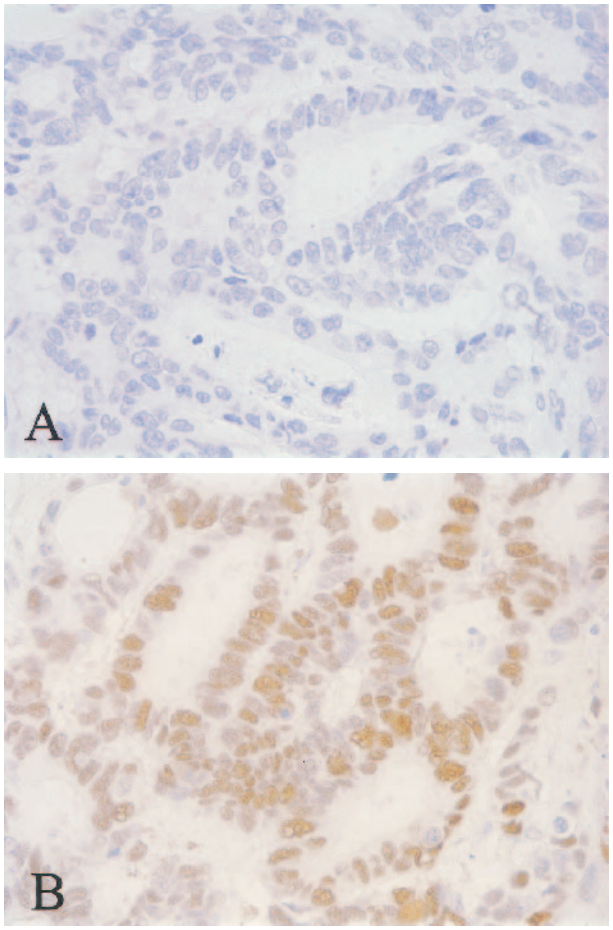

FIGURE 1. Representative examples of p53 phenotypes using mAb PAb1801 and immunohistochemistry. A, Negative p53 phenotype: no evidence of positive immunostaining in the nuclei of tumor cells. B, Positive p53 phenotype: colorectal adenocarcinoma sample displaying strong immunoreactive nuclei (original magnifications ×400).

RESULTS

Altered Patterns of p53 Expression: Phenotype Studies

A positive score for p53 accumulation required a nuclear staining in at least 10% of the tumor cells. Applying this criterion, we observed that 56 of 100 (56%) tumors displayed a positive phenotype, whereas the remaining 44 tumors were categorized as having a p53-negative phenotype (Fig. 1). Of the 44 tumors with a negative phenotype, 29 lesions were scored as 0 (undetectable levels of staining), whereas 15 tumors exhibited positive nuclear staining in less than 10% of tumor cells. We also observed that normal colonic mucosa and inflammatory and mesenchymal cells in all tissues analyzed had nonreactive nuclei, serving as internal negative controls.

TP53 Mutation Status: Genotype Studies

Using PCR-SSCP and direct sequencing, we identified and characterized TP53 tumor-specific mutations in 68 (68%) cases. Table 2 summarizes results obtained from these genotype analyses. Of interest, we observed 5 cases that displayed double mutations, with the number of mutations thus totaling 73. Fifty-one of the 73 detected mutations were missense; the remaining 22 mutations included 11 nonsense, 1 splicing, 8 frameshift (5 deletions and 3 insertions), and 2 in-frame deletions (18 bp) (Fig. 2). There were 63 mutations that involved replacement of a nucleotide, including 53 transitions (46 mutations involving G:C to A:T, 5 A:T to G:C, and 2 G:C to C:G changes) and 10 transversions (9 mutations were G:C to T:A, and only 1 was a T:A to G:C replacement). Thirty-five of the 46 identified G:C to A:T transitions affected CpG sites.

We found that frequent mutations were harbored at the so-called *hot spots*, including 10 mutations affecting codon 273, 9 at codon 175, 8 at codon 248, and 4 at codon 245. Interestingly, no mutations were found involving another reported hot spot at codon 249.

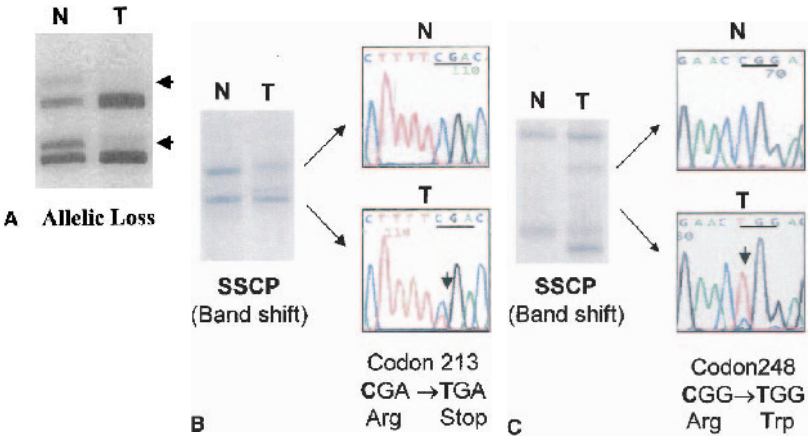

FIGURE 2. Representative cases of TP53 mutations using SSCP and sequencing assays. A, Allelic loss at the P53CA dinucleotide repeat. B, SSCP profile of exon 6: lane corresponding to normal tissue exhibits the 2 expected single-strand bands, whereas in the tumor sample, an anomalous mobility pattern, suspicious of a mutation, is identified. Sequence analysis of the shifted band showed a C to T change at codon 213 that resulted in a nonsense mutation. C, SSCP profile of exon 7: tumor lane displays an anomalous mobility pattern, and sequence analysis of the abnormal band showed a C to T change at codon 248 that resulted in a missense mutation. N, normal tissue; T, tumor tissue.

TABLE 2. *Spectrum of mutations*

Frequency	Exon	Codon	Change	Kind of mutation	IHC
1	4	38	CAA→TAA	NON	0
1	4	91	TGG→TGA	NON	>10%*
1	4	93	inser 1 nt (T)	FRA	>10%*
1	5	136	CAA→TAA	NON	0
1	5	163	TAC→TGC	MIS	>10%
1	5	174	AGG→AGT	MIS	>10%
9	5	175	CGC→CAC	MIS	>10%
1	5	176	TGC→TAC	MIS	>10%
1	5	179	CAT→TAT	MIS	>10%
1	5	152–156	del 13 nt	FRA	0
2	5	175–180	del 18 nt	DEL	>10%
1	5	175–180	inser 1 nt (C)	FRA	0
3	6	196	CGA→TGA	NON	0
1	6	203	GTG→TTG	MIS	>10%
2	6	213	CGA→TGA	NON	0; <10%**
1	6	195–201	del 19 nt	FRA	0
1	6	214	del 2 nt (TA)	FRA	0
1	7	237	ATG→ATA	MIS	>10%
1	7	241	TCC→TAC	MIS	>10%
2	7	245	GGC→AGC	MIS	>10%
1	7	245	GGC→GCC	MIS	>10%
1	7	245	GGC→GTC	MIS	>10%
2	7	248	CGG→CAG	MIS	>10%
6	7	248	CGG→TGG	MIS	>10%
1	7	229	del 2 nt (TG)	FRA	0
1	7	254	del 1 nt (C)	FRA	0
1	7	intron 7	G→T	SPLICING	0
1	7	240	Inser 1 nt (T)	FRA	0
1	8	264	CTA→CGA	MIS	>10%
2	8	266	GGA→GAA	MIS	>10%
1	8	266	GGA→GTA	MIS	>10%
2	8	272	GTG→ATG	MIS	>10%
6	8	273	CGT→CAT	MIS	>10%; <10%***
1	8	273	CGT→CCT	MIS	>10%
1	8	273	CGT→CTT	MIS	>10%
2	8	273	CGT→TGT	MIS	>10%
1	8	274	GTT→GCT	MIS	>10%
1	8	275	TGT→TAT	MIS	>10%
2	8	282	CGG→TGG	MIS	>10%
1	8	286	GAA→GGA	MIS	>10%
2	8	294	GAG→TAG	NON	0; <10%
2	8	297	CAC→CGC	MIS	>10%
1	8	306	CGA→TGA	NON	0

* A second mutation detected in the same case may account for the immunoreactivity.

** One sample was scored "0" and one <10%.

*** One sample was scored <10% and five were >10%.

We did not identified any mutation by direct sequencing in the 10% of cases (n = 3) analyzed as internal technical controls, which were randomly selected from the pool of tumors showing normal mobility patterns by PCR-SSCP (n = 32).

Comparison between p53 Phenotype and TP53 Genotype

TP53 gene mutations were detected in 51 of the 56 (91%) cases displaying a p53-positive phenotype by IHC. Of interest, all 5 tumors harboring double mutations were found to be associated with the p53-positive phenotype. Together, the mutations associated with detection of nuclear immunoreactivities included 50 missense mutations, 3 nonsense, 2 in-frame deletions (18 bp), and 1 frameshift.

Only 2 mutations in the group scored as less than 10% immunostaining and thus were considered p53-negative phenotype. Of these 2 mutations, 1 was a missense, and the other was a nonsense. Moreover, we found that of the 29 tumors scored as undetectable p53 levels or 0, and 15 showed TP53 gene mutations, including 7 nonsense, 7 frameshift, and 1 splice gene mutation.

Analysis of TP53 Allelic Losses

Genomic instability was detected in 8 of 100 tumors, which were excluded from the deletion study, because allelic loss in such cases could not be assessed. As per

LOH status, heterozygosity was observed in 87 (95%) of the remaining 92 tumors. Allelic losses at the TP53 locus were found in 55 of 87 (63%) evaluable tumors, whereas the remaining 32 informative cases did not show differences in allelic patterns when comparing normal and tumor lanes (Fig. 2).

Comparison Between TP53 Genotype Status and TP53 Allelic Losses

The comparative study between TP53 gene mutations and TP53 allelic losses was feasible in 87 cases. Allelic losses were detected in 47 of 64 (73%) mutated cases. Within the group of 23 nonmutated tumors, only 8 (35%) showed deletions, whereas the remaining 15 cases displayed no changes in their allelic pattern.

Molecular and Clinicopathologic Correlations: Statistical Analyses

Statistical associations were observed between TP53 mutational status and tumor size ($P = 0.005$, χ^2), pathologic stage ($P = 0.038$, χ^2), and histologic grade ($P = 0.004$, χ^2). We also found a significant association between TP53 allelic status and nodal involvement ($P = 0.040$, χ^2). TP53 deletions were associated with tumor stage ($P = 0.034$, χ^2), because they were deletions more common in patients with advanced disease. In addition, there were significant differences in frequency when comparing tumor size and tumors harboring TP53 mutations, because we observed that lesions smaller than 3 cm displayed a higher incidence of mutation than those larger than 6 cm ($P = 0.003$, Fisher exact test). No other associations were observed from this series when analyzing clinicopathologic parameters and laboratory results.

Significant differences were found between p53 expression patterns and detection of TP53 mutations. p53-positive phenotype was strongly associated with the identification of gene mutations ($P < 0.001$, Fisher exact test), in particular those of missense type ($P < 0.001$, Fisher exact test). Similarly, a strong degree of dependence between variables was obtained when comparing the incidence of TP53 mutations and TP53 allelic losses. Statistically significant differences were found between tumors harboring mutations depending on deletion status ($P < 0.001$, Fisher exact test), trending those not to retain the wild-type allele.

The ROC curve plot of all possible pairs of sensitivity-specificity values shifted the cut-off level at which IHC is considered positive to obtain the better trade-off sensitivity (0.750 in this case) and specificity (0.156) at any value within the range of 2% to 15% of positive nuclear immunostaining. With the same purpose, the performance of a logistic-regression analysis gave a significant adjustment, with 29.7% expected variability. The confidence interval of prediction allowed picking up 15% as the optimal cut-off point, with the sensitivity remaining at the same level of 75%. The area under the ROC curve was also calculated as a measure of the probability that a randomly chosen mutated tumor was correctly rated with a greater suspicion than a randomly chosen nonmutated one.[41] This probability was estimated at 79.3%.

DISCUSSION

This study analyzed a series of primary colorectal adenocarcinomas to define the degree of concordance between 2 of the most commonly accepted approaches to evaluate p53 status and to ascertain the potential clinical relevance of identifying p53 altered genotypes or phenotypes.

Identification of altered patterns of p53 expression in this study was performed by IHC using the mAb clone PAb 1801, which recognizes both wild-type and mutant p53 proteins.[35] It is widely accepted that missense mutations within the TP53 gene often increase the half-life of the encoded mutant product, leading to a good correlation between nuclear detection of immunostaining and presence of gene mutations. However, alterations that result in intragenic deletions or truncation of the protein have been reported to cause undetectable p53 immunostaining and accounting for a decreased sensitivity of IHC.[43] However, a PCR-based technique such as the SSCP followed by direct sequencing of altered bands may also render false results because of methodologic restrictions. The failure of some mutant forms to show abnormal patterns of mobility in gel electrophoresis,[44] and other limitations related to fragment length and sensitivity,[45] have been reported as contributing to false-negative data. Despite these limitations, it is widely accepted that the evaluation by SSCP of exons 5 to 8 of the TP53 gene, which contains more than 90% of all mutations occurring in primary tumors, followed by direct sequencing, is the method of choice for their identification.[4,25]

Data obtained from the comparative analyses conducted in this study reveal that although the p53-positive phenotype was strongly associated with detection of TP53 gene mutations ($P < 0.001$, Fisher exact test), the approaches have serious discrepancies. First, 15 of 29 tumors scored as having undetectable levels or 0 staining using IHC contained TP53 nonmissense mutations. These results suggest that in colon cancer, IHC by itself does not provide an accurate assessment of the functional p53 status. A second discrepancy was the lack of genetic evidence to support the finding of positive immunostaining in 5 of 56 tumors scored as having more than 10% positive cells. Mutations outside exons 4 through 8 may account for these inconsistencies, because it has been previously reported that as many as 10% of mutations can occur outside these exons in colorectal carcinoma.[4] However, the majority of these mutations generate stop

codons or frameshifts, which also produce negative IHC staining.[43] Technically, we can not rule out the possibility that some of the primers used in this study, which expand exonic sequences, missed mutations close to the intron–exon boundaries. In fact, direct sequencing of the same regions (exons 4 through 8) of SSCP-negative but IHC-positive cases evidenced a mutation in 1 case, missed by the PCR-SSCP approach (data not shown). Finally, another discrepant result is that observed for tumors scoring less that 10% positive tumor cells ($n = 15$), for which mutations were only detected in 2 cases. The lack of identifying gene alterations accounting for single scattered, positively stained cells could be explained by the limits on sensitivity of the techniques used. It could also signify detection of overexpressed p53 wild-type proteins in response to cellular stress or DNA damage.

The mutations identified in this study involved mainly exons 5, 7, and 8, with codons 175, 245, 248, and 273 the most commonly affected. It is significant to note that no mutations were detected at codon 249 in our series, despite being one of the most common hot spots.[43,46,47] Codon 249 mutants have mainly been reported to occur in hepatocellular carcinoma, related to high dietary aflatoxin B1 exposure and hepatitis B virus infection. In our study, 35 of 63 (56%) mutations involving nucleotide replacement were G:C to A:T transitions at CpG sites. These mutations are likely to have arisen by spontaneous deamination of 5-methylcytosine. This fact, added to the lack of mutation occurring at codon 249, corroborates a high contribution of endogenous versus exogenous factors in our series of colorectal cancer.[3,32,43]

In order to validate IHC results as they relate to SSCP and sequencing data, we used a ROC analysis. This test provides a range of optimal values that are susceptible to being chosen as cut-off points, considering a reasonable balance between the 2 types of errors (false-negatives and false-positives). This range included all the IHC values between 2% and 15% that were associated with a sensitivity and specificity of 75% and 84%, respectively. The area under the curve may be considered a measure of the global accuracy of the IHC approach when referred to molecular results, independently of the choice of the cut-off value. In our series, this area was estimated to be 79.3%.

In relation to LOH results, 87 of 92 samples (95%) were informative for the P53CA polymorphism; these data were consistent with those previously published.[40] We found a strong association between p53 mutations and 17p allelic losses. Although this relation has been reported in other studies, monitoring of either alteration does not convey identical information. Indeed, there is no strict correspondence between these 2 parameters; p53 mutation and 17p allelic loss may not occur simultaneously or may not be directly related in some tumors. Some studies have suggested that when the histologic staging is not taken into account, determination of the allelic status of the tumor confers more prognostic information than detection of p53 mutation.[48] However, in clinical practice today, histologic staging provides the predominant prognostic information.

Advances in adjuvant chemotherapy for patients with colorectal cancer require determination of the patients that may benefit from such regimens, giving each individual patient a better chance for cure. In this regard, a predictive marker assessment would be of clinical significance, especially for those cases presenting with intermediate-grade tumors. In this respect, identification of a p53-positive phenotype and TP53 mutations was significantly associated with tumor aggressiveness, because these biologic determinations were related to advanced stage, high tumor grade, and positive lymph node status. It appears that monitoring p53 overexpression or mutation rather than p53 immunopositivity alone contributes best to this requirement.

In sum, data from this study suggest that assessment of p53 in colorectal tumors must be performed using a combination of techniques, including IHC and molecular assays. This approach may be even more critical when cases display absence of nuclear staining. The application of laboratory techniques in a clinical setting requires a reproducible assay that is widely available with quality control and is readily interpretable. The approach and methods described in this study provide details for the rapid and accurate identification of almost all p53 alterations using nonradioactive materials, facilitating such clinical processes. □

Acknowledgments: The authors thank Dr. Miquel Calvo Llorca from the Statistics Department, Facultat de Biologia, Universitat de Barcelona, for assistance regarding statistical analyses.

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