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p53 Gene status and expression of p53, mdm2, and p21Waf1/Cip1 proteins in colorectal cancer

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Abstract Abrogation of the normal p53 pathway is the most common molecular alteration in human cancer. *p53* Gene status can be potentially assessed through the expression of proteins known to be activated by the wild-type *p53* (*wt p53*) system, such as mdm2 and p21Waf1/Cip1. In this study, the frequency of mdm2, p21Waf1/Cip1, and p53 protein expression was investigated using immunohistochemistry (IHC) in 88 colorectal carcinomas (CRCs). The relationship between these expressions and *p53* status was examined. *p53* status and the immunophenotypes characterizing these tumors were correlated with standard prognostic variables. Mutation of *p53* was detected using single-strand conformational polymorphism (SSCP) analysis and sequencing. Concordance between *p53* gene status and p53 immunoreactivity was seen in 62 of 88 (70.45%) carcinomas. Mdm2 expression was found in 22 of 45 (48.88%) and 5 of 43 (11.62%) of the tumors with *wt p53* and mutated *p53* ($P<0.0001$), respectively. Predominantly, higher p21Waf1/Cip1 expression was associated with *wt p53* ($P<0.001$). All *wt p53* cases that expressed mdm2 also expressed p21Waf1/Cip1. These results suggest that there is a subgroup of CRCs in which p53 is functionally active, inducing transcription of *mdm2* and *Waf1/Cip1*. Their combined evaluation may provide important clues for planning adjuvant systemic therapy and gene therapy based on the restitution of *p53* function. However, no significant association was found between the immunophenotypes and the standard prognostic variables investigated.

Keywords Colorectal carcinoma · p53 · mdm2 · p21Waf1/Cip1 · SSCP

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Introduction

The human *p53* gene is a well-characterized tumor suppressor gene. *p53* mutations have been found in several types of human cancer. It has been recorded as the most common genetic event associated with human malignancy. On the genetic level, the mechanisms of inactivation of *p53* are missense mutations in one allele and a gross deletion of all or a large portion of the remaining wild-type (wt) allele of human chromosome 17 [2, 17]. In colorectal carcinomas (CRCs), alterations of the *p53* gene are reported to occur in 40–70% of the cases [20], playing an important role in carcinogenesis [12, 19, 30]. p53 is involved in gene transcription, cell cycle control, and apoptosis. Functional inactivation of *p53* by mechanisms other than mutation has been described [22, 23]. It is known that the wt but not the mutant p53 protein can positively regulate the *mdm2* gene transcription in an autoregulatory feedback loop [3, 34]. The mdm2 protein modulates the activity of p53 by forming a protein complex with p53, which can result in the inactivation of p53 tumor suppressor function [23].

Wt p53 regulates the G0-G1 checkpoint and, in response to DNA damage, can induce cell-cycle arrest through transcriptional activation of the *Waf1/Cip1* gene or by apoptosis [28, 31]. It was shown that in tumor cells that contain an altered form of p53, p21 levels are greatly reduced or totally absent [10], leading to an abnormal control of the cell-cycle progression.

Prompted by these data, we investigated the p53, mdm2, and p21Waf1/Cip1 protein expression as assessed using immunohistochemistry (IHC) and compared the

Table 1 Primer sequences of exons 4–9 of the *p53* gene and polymerase chain reaction (PCR) conditions. *Ann Temp* annealing temperature

Exon	Sense	Antisense	Product length (bp)	Ann temp (°C)	$MgCl_2$ (mmol/µl)
4	atctacagtccccccttgccg	gcaactgaccgtgcaagtca	295	58	1.5
5	gactttcaactctgtctcctt	accagccctgtcgtctctccg	253	55	1.5
6	aggcctctgattcctcactga	ccagagaccccagttgcaaac	170	55	1.5
7	aaggcgcactggcctcatctt	gcacagcaggccagtgtgcag	192	55	1.5
8	aggacctgatttccttactgc	tgcacccttggtctcctccac	231	55	1.5
9	cctatcctgagtagtggtaa	ccaagacttagtacctgaat	331	60	3.5

results with the *p53* gene status in a series of 88 CRCs. The distributions of p53, mdm2, and p21Waf1/Cip1 proteins were examined in order to more precisely characterize *p53* gene status compared with immunohistochemical p53 nuclear reactivity. p21Waf1/Cip1 and mdm2 protein expression could potentially reflect the functional status of *p53* in CRCs. The potential clinical and biological implication of the immunophenotypes characterizing these tumors was assessed.

Materials and methods

Eighty-eight sporadic, primary colorectal adenocarcinoma specimens routinely submitted to our department from December 1994 to September 1997 were investigated. Fifty-two (59%) patients were male and 36 (40.9%) were female, with ages ranging from 24 years to 87 years [average age 65.0±1.2 years (mean±SEM)]. No initial chemotherapy or radiotherapy was given before tumor excision. Both tumor tissue and normal mucosa from each case were snap-frozen and stored in liquid nitrogen until required. The remainder of the tumor sample was fixed in 7.5% neutralized formalin and embedded in paraffin. One or two representative paraffin blocks were selected, generally including the greatest diameter of the lesion, from which serial sections were examined using light microscopy and IHC. Clinical and pathological stage was assessed following the UICC TNM staging system [15] and the Dukes' A–D classification according to the Erlangen modification of the Dukes original system [9, 16].

Screening for *p53* mutations employed established polymerase chain reaction (PCR)-based methods in combination with single-strand conformational polymorphism (SSCP) analysis in all 88 cases. Paired samples of genomic DNA, from normal and from the corresponding colorectal cancer tissue specimens, were extracted using a QIAamp tissue protocol (QIAGEN GmbH, Hilden Germany, 1997). Segments of the *p53* gene, corresponding to exons 4–9 were amplified separately. PCR conditions and primer sequences are outlined in Table 1.

SSCP analysis was used for screening PCR amplicons from exons 4–9 of tumor and mucosal DNA from each patient. SSCPs were run on 8–10% polyacrylamide gels with 10% glycerol overnight at 4°C. Gels were stained using the silver stain plus kit (Bio-Rad Laboratories, Hercules, Calif.). PCR products from tumors with *p53* mutations detected on SSCP and IHC negative and with less than 10% positivity, and tumors without *p53* mutations and IHC of 10% or more were further subjected to DNA sequencing.

Before sequencing, PCR amplicons were purified with the Talent Clean Mix Kit (Talent, Trieste, Italy). A DNA sequencer Li-Cor Model 4000L (Li-Cor, Lincoln, Neb.) was used for cycle sequencing with 5'-IRD 41-labelled nested forward (5'→3') and reverse (3'→5') primers and the Thermo Sequenase Cycle Sequencing Kit (Amersham, Aylesbury, UK).

To analyze p53, p21, and mdm2 expressions, formalin-fixed (7.5% buffered formalin), paraffin-embedded tissue sections (4 µm) were used, deparaffinized, and exposed to an antigen retrieval system before being incubated with the respective antibodies (citrate buffer, pH 6, and microwave radiation). The avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Calif.) was performed using monoclonal antibodies (mAb) raised against the following antigens as primary Abs: mdm2 (Ab1, IF2; diluted 1:50; Calbiochem, Calif.); p53 (DO-1 Ab; diluted 1:20; Immunotech SA, Marseille, France); p21Waf1 (diluted 1:20; Calbiochem, Calif.).

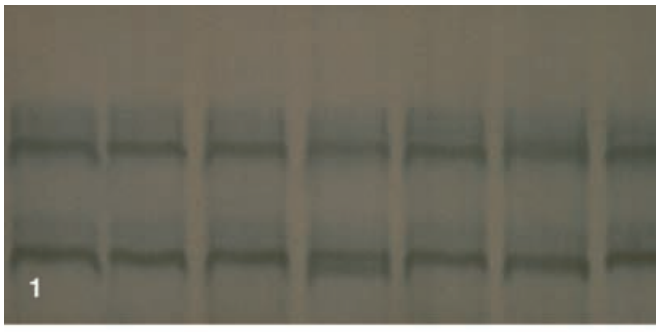
Nuclear immunoreactivity for p53 and p21Waf1/Cip1 were graded semi-quantitatively [0 (0%); 1+ (1–9%); 2+ (10–50%); 3+ (>50%)]. Mdm2 was scored as positive (any positivity) or negative (no staining). Due to considerations concerning the numerical aspect of p53 overexpression [14], a 10% cut-off value was adopted. All of the IHC analyses were performed blinded to each other and to the *p53* results.

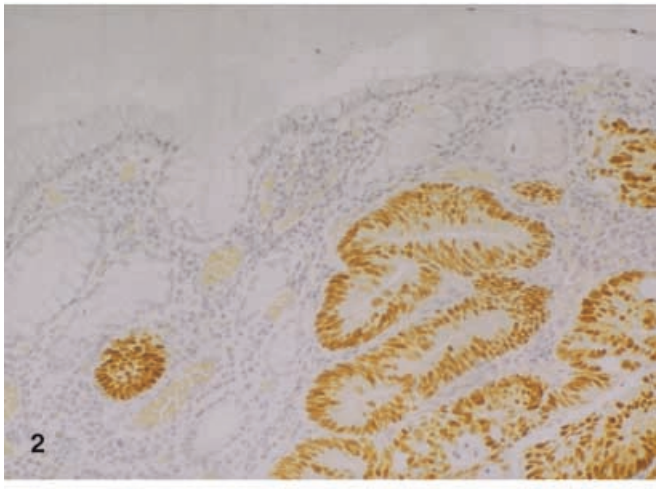
The relationships between *p53* gene status and/or the immunohistochemical results and the different pathological and clinical variables were tested using χ^2 or Fisher's exact test, when appropriate. Continuous variables were analyzed using the Wilcoxon's two-sample test. Two-sided *P* values less than 0.05 were considered significant. SPSS statistical software was used for analysis.

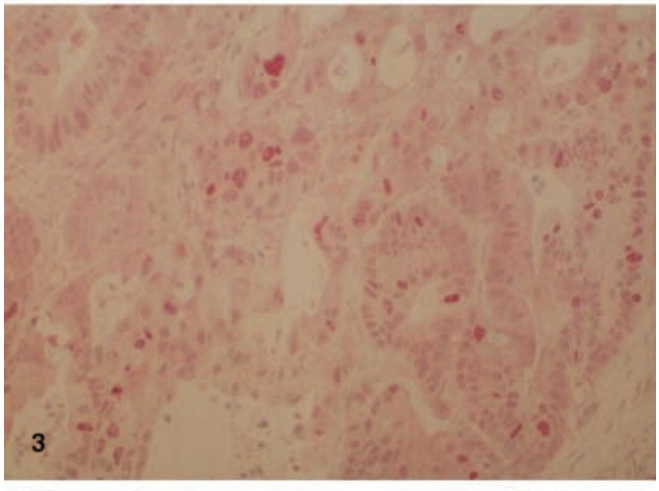
Results

In the group of 88 CRCs investigated (45 wt and 43 mutated *p53* genes), there were 27.9% (12/43) mutations in exon 8 (Fig. 1), 25.58% (11/43) in exon 7, 20.9% (10/43) in exon 6, 18.6% (8/43) in exon 5, 4.65% (2/43) in exon 9, and one case (2.32%) showed mutations in exons 5 and 7. No mutation was found in exon 4.

The pattern of p53 immunostaining in positive cases was heterogeneous, with a widespread positivity over segments of the tumors, the entire sections, or with scattered positive nuclei throughout the tumors (Fig. 2). Overall, 74 of 88 (84.1%) of the CRCs in this study exhibited positive p53 nuclear staining, 37 of 45 (42.05%) of them corresponding to the wt cases and 37 of 43 (42.05%) corresponding to the cases with a mutation in the *p53* gene. By adopting a 10% positivity cut-off value, the concordance between *p53* gene mutation and p53 immunoreactivity (i.e., both negative or both positive, with immunostaining ≥10% positive cells) was seen in 62 of 88 (70.45% $P<0.0001$). A further 8 of 88 (9.09%) of the carcinomas showed mutant bands using SSCP analysis and no immunostaining, while 18 of 88 other tumors (20.45%) exhibited positive staining and no mutant bands (Table 2). Five of the eight cases of SSCP+/IHC– were sequenced. Two silent mutations, two stop codons, and one frameshift mutation were detected. All of the 18 tumors that were SSCP–/IHC+ were also sequenced, and no mutations could be detected (Table 3). None of the clinical and pathological parameters analyzed, except







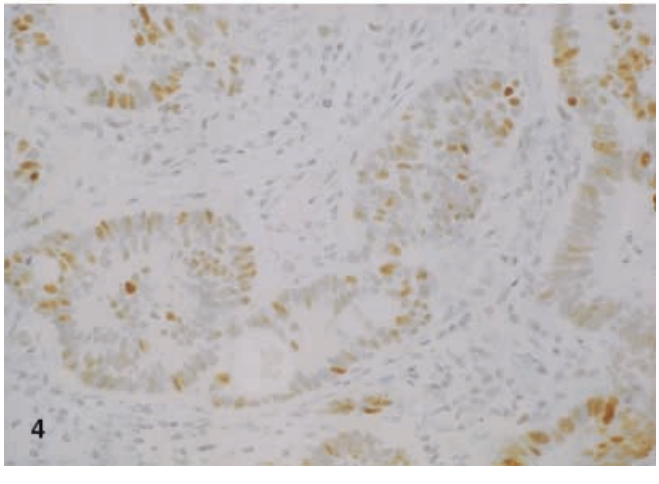


Table 2 Relationship between p53 immunohistochemistry and *p53* gene status. *SSCP* single-strand conformational polymorphism; *IHC* immunohistochemistry

	SSCP wild-type *p53*	SSCP mutated *p53*	Total
IHC <10%+	27 (30.68%)	8 (9.09%)	35 (39.77%)
IHC ≥10%+	18 (20.45%)	35 (39.76%)	53 (60.23%)
Total	45 (51.13%)	43 (48.86%)	88 (100%)

gender, correlated with p53 protein expression or the *p53* gene status (Table 4). Although there was an association between *p53* gene status and/or p53 protein expression and tumor site and Dukes' stage, it did not achieve statistical significance.

Mdm2-positive nuclear staining was found in 22 of 45 (48.88%) of the cases with *wtp53* and in 5 of 43 (11.62%) of the patients with a mutated *p53* gene ($P<0.0001$). Detailed data are listed in Table 5. Mdm2 expression was observed in 19 of 35 (54.28%) of the cases with p53 expression with less than 10% positivity and in 8 of 53 (15.09%) of the cases with overexpression of p53 ($P<0.0001$). Immunoreactivity for mdm2 in tumor cells was always nuclear. Normal epithelial cells showed some degree of faint cytoplasmic staining, and stromal fibroblasts and lymphocytes did not show any immunostaining. Positive tumor nuclei were scattered in the neoplastic tubules and corresponded to no more than 7% of the carcinoma cells (Fig. 3).

p21Waf1/Cip1 nuclear staining was found in 39 of 45 (86.66%) and 35 of 43 (81.39%) of the cases with a wt and mutated *p53* gene, respectively. In the group with *wtp53* gene, 29 of 45 (64.44%) of the cases showed extensive expression of p21Waf1/Cip1, while in the group of mutated *p53*, there was reduced or no expression in all cases ($P<0.001$). In only 7 of 43 (16.27%) *p53*-mutated carcinomas, there was total concordance between *p53* gene status, p53 protein overexpression, and lack of p21Waf1/Cip1 expression. Detailed data are listed in Table 5. Positive nuclei were observed scattered in single tumor glands or in large segments of the tumors (Fig. 4). In addition, positive nuclei were found in the upper crypts and surface epithelium of the non-neoplastic colorectal mucosa.

There was concordant expression of mdm2 and p21Waf1/Cip1 in 22 of 45 (48.88%) of the *wtp53* cases. All *wtp53* cases that expressed mdm2 protein also ex-

Fig. 1 Polymerase chain reaction (PCR)/single-strand conformational polymorphism (SSCP) analysis of exon 8 of the *p53* gene. *Lanes 1*, *3*, and *5* Single strands of normal mucosa samples; *lanes 2*, *4*, and *6* single strands of the corresponding tumor samples of three different patients. *Lane 4* shows a band shift indicating a mutation

Fig. 2 Immunostaining of p53 with a widespread positivity of tumor nuclei (3+ positivity; mutated *p53* case)

Fig. 3 Mdm2-positive immunostaining in a case with wt *p53*

Fig. 4 Immunostaining of p21Waf1/Cip1 in a case with wt *p53* (2+ positivity)

Table 3 Colorectal carcinomas with discordant single-strand conformational polymorphism (SSCP)/immunohistochemistry (IHC) results

No.	SSCP	p53 IHC	p53 Sequence analysis			mdm2 IHC	p21 IHC
			Base	Codon	Amino acid		
1	Exon 7	0	Not done			0	1
2	Exon 6	0	cgg→tga	213	Arg→stop	0	0
3	Exon 8	0	cga→tga	306	Arg→stop	0	2
4	Exon 7	0	ctc→-tc	252	Frameshift	0	1
5	Exon 7	0	Not done			0	1
6	Exon 5	0	Not done			0	1
7	Exon 6	1+	cgg→cga	213	Arg→Arg	1	1
8	Exon 6	1+	cgg→cga	213	Arg→Arg	1	2
9	–	2+	No mutation detected			0	2
10	–	2+	No mutation detected			0	1
11	–	2+	No mutation detected			1	3
12	–	2+	No mutation detected			0	2
13	–	2+	No mutation detected			0	1
14	–	2+	No mutation detected			1	2
15	–	2+	No mutation detected			1	2
16	–	2+	No mutation detected			0	2
17	–	2+	No mutation detected			1	2
18	–	2+	No mutation detected			1	3
19	–	2+	No mutation detected			0	2
20	–	3+	No mutation detected			0	2
21	–	3+	No mutation detected			0	2
22	–	3+	No mutation detected			1	3
23	–	3+	No mutation detected			0	2
24	–	3+	No mutation detected			0	0
25	–	3+	No mutation detected			0	0
26	–	3+	No mutation detected			0	0

Table 4 Relationship of *p53* gene status and clinico-pathological and biological parameters

Variable	Wild-type *p53* No. (%)	Mutated *p53* No. (%)	*P* value[b]
Age[a]	66 (24–87)	66 (44–85)	0.540
Gender			
Male	21 (46.7)	31 (72.1)	0.015
Female	24 (53.3)	12 (27.9)	
Site			
Right colon	24 (53.3)	15 (34.9)	0.058
Left colon	7 (15.6)	16 (37.2)	
Rectum	14 (31.1)	12 (27.9)	
Grading			
G1	3 (6.7)	3 (7.0)	0.570
G2	38 (84.4)	33 (76.7)	
G3	4 (8.9)	7 (16.3)	
Dukes' stage (Erlangen modification)			
A	6 (13.3)	5 (11.6)	0.669
B	19 (42.2)	13 (30.2)	
C1	11 (24.4)	14 (32.6)	
C2	5 (11.1)	8 (18.6)	
D	4 (8.9)	3 (7.0)	
Tumor depth			
pT1	2 (4.4)	2 (4.7)	0.980
pT2	6 (13.3)	6 (14.0)	
pT3	31 (68.9)	28 (65.1)	
pT4	6 (13.3)	7 (16.3)	

[a] Median (range)
[b] Significant: $P<0.05$

pressed p21Waf1/Cip1. However, in the cases with mutated *p53*, only seven (16.27%) were concordant negative for mdm2 and p21Waf1/Cip1 expressions (Table 5 and Table 6). As already mentioned, only low intensity staining of p21Waf1/Cip1 was associated with the *p53* mutation.

No significant correlation was observed between p53 (SSCP/IHC) and different expressions of mdm2 and p21Waf1/Cip1 and any of the clinical, pathological, and biological parameters investigated. Results for *p53* gene status are summarized in Table 4.

Table 5 Relationship between *p53* gene status and p21Waf1/Cip1 and mdm2 protein expression

	Wild-type *p53*	Mutated *p53*	*P* value
Mdm2+ expression	22/45 (48.88%)	5/43 (11.62%)	0.0001
p21Waf1/Cip1 expression			
0%	6/45 (13.33%)	8/43 (18.6%)	0.001
1–9%+	10/45 (22.22%)	24/43 (55.81%)	
10–50%+	24/45 (53.35%)	11/43 (25.58%)	
>50%+	5/45 (11.11%)	0	

Table 6 *p53* Gene status and patterns of p53, mdm2, and p21 protein expression

p53 Status	Patterns	No. of cases
Wild-type *p53*	p53–/mdm2+/p21+[a]	16
	p53–/mdm2–/p21+[a]	8
	p53–/mdm2–/p21–[a]	3
	p53+/mdm2–/p21+	9
	p53+/mdm2+/p21+	6
	p53+/mdm2–/p21–	3
Mutated *p53*	p53+/mdm2–/p21+[a]	26
	p53+/mdm2–/p21–[a]	6
	p53+/mdm2+/p21–[a]	1
	p53+/mdm2+/p21+[a]	2
	p53–/mdm2–/p21+	5
	p53–/mdm2–/p21–	1
	p53–/mdm2+/p21+	2

[a] Cases with concordant p53 (single-strand conformational polymorphism (SSCP)/immunohistochemistry (IHC)

Discussion

Most forms of mutations of the *p53* gene result in the production of abnormal proteins with a prolonged half-life. IHC has been relatively effective in identifying missense point mutations that are located in the highly conserved domains in exons 5–8 [5]. These mutations are by far the most common *p53* genetic alterations [17]. Thus, IHC has been proposed as an indirect method of p53 mutation screening in several types of human neoplasias. The relationship between *p53* mutations and p53 protein accumulation is variable, and several authors have reported discrepant results. In CRCs, this association was referred to be present in 70% of the cases [5, 8, 21]. In the present work, the expression patterns of p53, p21Waf1/Cip1, and mdm2 proteins were investigated in relation to *p53* gene status. The combined expressions of these proteins were evaluated in order to more precisely characterize the *p53* gene status compared with p53 IHC alone. The wt, but not the mutant p53 protein, can positively regulate the mdm2 gene transcription in an autoregulatory feedback loop [3, 34]. Mdm2 expression can therefore indicate a normally functioning p53 system [18, 33]. However, the *Waf1/Cip1* gene can be activated through two separate pathways: a p53-dependent one activated by DNA damage and another p53-independent one mainly related to cell differentiation [10, 11, 31, 36]. p21Waf1/Cip1 immunoreactivity has recently been described in human neoplasms. p21Waf1/Cip1 expression could potentially reflect the functional status of p53 in p53-dependent tumors. Regarding CRCs, some reports in the literature have yielded discrepant results about the relationship between p53 and p21Waf1/Cip1 [13, 26, 35]. Therefore, in the present study, the combined expression of mdm2 and p21Waf1/Cip1 was analyzed in relation to *p53* gene status as assessed using SSCP analysis and IHC to find a preserved function of *p53*. In addition, the potential clinical implication of the immunophenotypes characterizing subgroups of these tumors was assessed.

We found p53 nuclear immunoreactivity in 74 of the 88 cases (84.1%), 37 (42.05%) corresponding to the wt, and 37 (42.05%) to the cases with a mutation in the *p53* gene. By adopting a 10% positivity cut-off value, the concordance between *p53* gene mutation and p53 IHC (both negative or SSCP–/IHC <10% positivity; or SSCP+/IHC ≥10% positive cells) was seen in 62 of the 88 cases (70.45%). This is similar to the results reported in previous studies. Several explanations could account for the discrepancies between *p53* gene status and p53 protein expression, including the occurrence of nonsense and frameshift mutations that do not lead to protein stabilization [5], the antibody selection and tissue fixation [1, 14], and other factors that contribute to p53 protein stabilization [6, 25, 27]. In this study, 35 of the 53 (66.03%) cases with p53 IHC of 10% or more had a *p53* gene mutation. Eighteen of these cases with IHC of 10% or more did not show altered bands using SSCP analysis. All of those 18 cases were further sequenced, and no mutations were found in the *p53* gene. Twelve of these cases (66.66%) did not express the mdm2 protein. However, p21Waf1/Cip1 expression was observed in 15 of them (83.33%) and in more than 50% of the neoplastic cells in 3 of these 15 cases. In those cases, it is concluded that mechanisms other than mutation of the *p53* gene are responsible for p53 accumulation and functional inactivation, even in the presence of a wt *p53* gene [4, 18]. p21Waf1/Cip 1 expression may also be regulated by mechanisms independent of p53.

Eight of the tumors (22.22%) with *p53* gene mutations had null or less than 10% reactivity for p53 IHC. Five of these eight cases of SSCP+/IHC– were sequenced. Two silent mutations, two stop codons, and one frameshift mutation (Table 3) were detected. As referred, non-sense and frameshift mutations do no lead to protein stabilization. Only those cases with silent mutations expressed mdm2. Low or no expression of p21Waf1/Cip1 was observed in all except one case.

The significant association between mdm2 and p53 expression/gene status in our study suggests that, in those cases, *p53* is functionally active, and mdm2 positivity represents a functional mdm2 protein. Positive tumor nuclei were found scattered in the neoplastic tubules similar to the p53 immunoreactivity, suggesting co-expression of the two proteins in individual cells [18]. However, mdm2 was also expressed in 5 of the 43 cases with mutated *p53*, suggesting that other mechanisms might be inducing mdm2 expression in CRCs [25]. In these cases, only a few single positive cells were observed using IHC. Concordant expression of p21Waf1/Cip1 and mdm2 was noted in 18/62 (29.03%) of the cases with concordant p53; 16 of them were SSCP–/IHC–, and the other two cases (SSCP+/IHC+) were the ones which showed silent mutations. Within the group of tumors with discordant p53, only 4 of the 25 (16%) were concordant negative for mdm2 and p21 expression. Although p21Waf1/Cip1 protein is induced by the *wtp53* gene, our results indicate that p21Waf1/Cip1 expression might be regulated by p53-dependent and independent pathways in this neoplasia, as previously reported [24, 26, 29, 32, 35] and also referred in other types of carcinomas [7]. However, our results did not show any significant association between p53, p21Waf1/Cip1, and mdm2 protein expression with tumor grade, and pT and Dukes' stage, as also referred by others [13, 26]. However, other studies showed an inverse relationship between p21 expression and Dukes' stage or tumor depth [30, 35]. The positive correlation between gender and p53 gene status is probably coincidental.

In conclusion, our results show that there is a highly significant correlation ($P<0.0001$) between *p53* gene status and p53 expression as detected using IHC. Most important, although not all cases with wt *p53* seem to have a functioning p53 system, there is a subgroup of CRCs of which p53 is functionally active, inducing transcription of *mdm2* and *Waf1/Cip1*. This suggests that p53-independent mechanisms are associated with uncontrolled cell growth and cancer development in the large bowel, providing some important clues for planning future gene therapy and chemoradiotherapy. However, accumulation of p53 protein might indicate either the presence of an underlying gene mutation in most cases or p53 inactivation. Negative p53 IHC, although most frequently associated with the wt *p53* gene and functionally active p53, may also hide undetected p53 mutations or p53 inactivation.

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