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p21 Expression in colorectal carcinomas: a study on 103 cases with analysis of p53 gene mutation/expression and clinic-pathological correlations

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Abstract The WAF1/CIP1 gene product, p21, an inhibitor of cyclin-dependent kinases, is a critical downstream effector in the p53 pathway. The expression of p21 in human neoplasms is heterogeneous, and may be related to p53 functional status. We evaluated p21 immunoreactivity in 103 colorectal carcinomas (CC) in relation to the p53 gene and protein alterations and clinico-pathologic parameters. High p21 expression (more than 10% reactive cells) was seen in 39% of cases. p21 staining was heterogeneous and often detected in clusters of tumour cells; in some tumours p21 staining was more pronounced in superficial areas. No relation was seen between p21 immunoreactivity and site of the tumours (right vs left), TNM stage and grade. p21 expression was related to p53 status as evaluated with IHC or with SSCP analyses, low p21 expression usually being associated with p53 protein overexpression (P=0.048) and p53 gene alteration (P=0.005). The strongest associations were seen when the combined p53/p21 immunophenotype was compared with p53 gene alterations (P=0.0002). These data support the hypothesis that p21 expression in CC is mainly related to p53 functional status, suggesting that p21 expression could be an interesting adjunct in the evaluation of the functional status of the p53 pathway in CC.

Key words p21 · p53 · Colon · Immunohistochemistry · Human

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Introduction

Colorectal cancer (CC) is a sporadic and familial condition characterised by imbalance between cell growth, apoptosis and differentiation, associated with the accumulation of genetic alterations over a period of many years [8, 18, 21, 33]. Alterations in the p53 tumour-suppressor gene are among the most frequently encountered genetic aberrations in CC [24]. Most p53 gene alterations are mutations involving exons 5–8, which can be investigated with single-strand conformational polymorphism (SSCP) analysis. Most mutations stabilise p53 gene product, which accumulates in the cell nuclei and can be detected by immunohistochemistry, and there is a well-known association between p53 gene mutation and p53 protein overexpression [2].

The wild-type p53 protein suppresses tumour cell growth, binds to specific DNA sequences and participates in cell-cycle regulation by transactivating several genes, which include MDM2, Gadd 45 and WAF1/CIP1. The WAF1/CIP1 gene product, p21, is a general inhibitor of cyclin-dependent kinases (CDKs), which regulate entry into the DNA synthesis phase of the cell cycle [14]. p21 expression due to wild-type p53 overexpression in response to DNA damage causes G1 growth arrest through inhibition of CDKs, and may also be involved in G2 arrest [7]. However, apart from induction by wild-type p53, activation of the WAF1/CIP1 gene can also occur through mechanisms independent of p53, mainly related to cell differentiation [15, 46]. Indeed, p21 may play an important part in the maintenance of growth arrest in terminally differentiated cells.

p21 immunoreactivity has recently been observed in human neoplasms, where it is differently associated with p53 integrity depending on tissue type: in some tumour types p21 is mainly p53 regulated, while in others it is mainly regulated through p53-independent pathways. In tumours where p21 expression is mainly induced by wild-type p53 protein, the level of p21 itself could potentially reflect the functional status of p53 in cancer cells, which in turn could be of potential clinical interest [6, 40].

Table 1 Relation between the clinic-pathologic parameters and p53 and p21 alterations (*IHC* immunohistochemical staining of tumour cells, expressed as percentage of reacting cells, *SSCP* p53 gene alteration assessed using the nonradioactive PCR-SSCP (single-strand conformation polymorphism) analysis with primers for exons 5, 6, 7, 8; nonpolymorphic electrophoretic band shifts were considered to indicate p53 gene mutation)

Tumour parameters	No.	p53 IHC ≥40%	p53 IHC <40%	p53 SSCP		p21 IHC ≥10	p21IHC <10
				With band shift	Without band shift		
Stage							
T2	26	15	11	14	12	7	18
T3	62	39	23	27	35	21	37
T4	8	4	4	3	5	3	5
Tx	7						
Grade							
1	5	1	4	1	4	4	1
2	60	36	24	28	32	18	39
3	30	18	12	12	18	10	18
Gx	8						
Metastases							
N0	54	37	17*	27	27	17	33
N1/2/3	46	22	24	18	28	14	27
Nx	5						
Site							
Right colon	29	14	15	12	17	12	17
Left colon	63	40	23	33	30	17	42
Unknown	11						

*P=0.07 Chi-square with Yates correction, P =0.046 Mantel

The potential interest on p21 expression in CC is further enhanced by the fact that the levels of p21 can be manipulated, at least in cell cultures, by some drugs, including retinoids [27] and sulindac, a nonsteroidal agent that lowers the incidence and mortality from CC [20]. In CC p21 is heterogeneously expressed, and preliminary data suggest that its expression is regulated mainly by p53 [13, 15] and might be of possible prognostic value. There are, however, some studies in the literature that have yielded discrepant results regarding the relation between p53 and p21 in CC [41]: therefore, we undertook the present study to investigate the immunohistochemical expression of p21 in relation to p53 gene and protein alterations and clinic-pathologic features further in a large series of patients with CC.

Materials and methods

We evaluated 103 formalin-fixed paraffin-embedded specimens from cases of sporadic CC diagnosed and treated at S. Chiara Hospital in Trento in 1996–1997. Clinical and pathological staging was performed according to the UICC TNM system. Pertinent data on the tumours are summarised in Table 1 (first column).

Tumour samples were immediately fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin. On the basis of H&E-stained sections, the most representative tissue block for each sample was selected for immunohistochemical studies and DNA extraction. An additional sample of histologically normal mucosa at the margins of the surgical samples (at least 10 cm away from the tumour) was selected to extract normal DNA for each patient.

For immunohistochemistry, 5-µm sections were cut from paraffin blocks and mounted on xylanated slides. Expression of p21 was evaluated using the EA10 monoclonal antibody (Oncogene Science, Cambridge, Mass.) diluted 1:100 after microwave treatment, as described elsewhere [3, 13]. Positive controls were sections of lung tumours known to express p21 at the mRNA and protein levels [31]. Surface epithelium of the normal mucosa adjacent to tumours was regarded as a positive internal control [13]. Negative controls were obtained by omitting primary antibody. Immunoreactivity was scored by evaluating at least 500 tumour cells for nuclear staining; cases with immunoreactivity in 10% or more were considered to express high values of p21. Expression of p53 has been evaluated with D07 monoclonal antibody, with 1:100 dilution after microwave treatment, as described elsewhere [3]. Immunoreactivity was scored by evaluating at least 500 tumour cells for nuclear staining; cases with immunoreactivity in 40% or more were considered to overexpress p53.

Genomic DNA was extracted from the paraffin-embedded tissues. Sections were dewaxed in xylene and alcohol and treated with proteinase K 400 (ng/µl) in 250 µl of digestion buffer (50 mM Tris HCl, pH 8; 5 mM EDTA, pH 8; 0.5% Tween 20) and incubated overnight at 55°C. Proteinase K was heat inactivated, and the samples were used directly for PCR. Exons 5 through 8 of the p53 gene were amplified using four pairs of specific intronic oligonucleotides primers [30]. The size of PCR products were as follows: exon 5, 290 bp; exon 6, 206 bp; exon 7, 214 bp; and exon 8, 240 bp. The PCR mix consisted of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM of dNTPs, and 0.5 µM of each primer. For the template, 1 µl of the digestion mixture was used. The PCR temperature profile for exons 6, 7, and 8 was as follows: (a) denaturation at 94°C for 1 min, (b) annealing for 1 min at 63°C, and (c) extension for 1 min at 72°C, for a total of 39 cycles. The extension for the last cycle was increased to 3 min to ensure complete extension. For exon 5 the temperature of annealing was 57°C for 1 min. A negative control containing no DNA was included in each PCR experiment. The amplification products were visualised on an ethidium bromide-stained 2% Pharmacia gel. For single-strand conformation polymorphism (SSCP) analysis, PCR products (4 µl) were mixed with 10 µl of

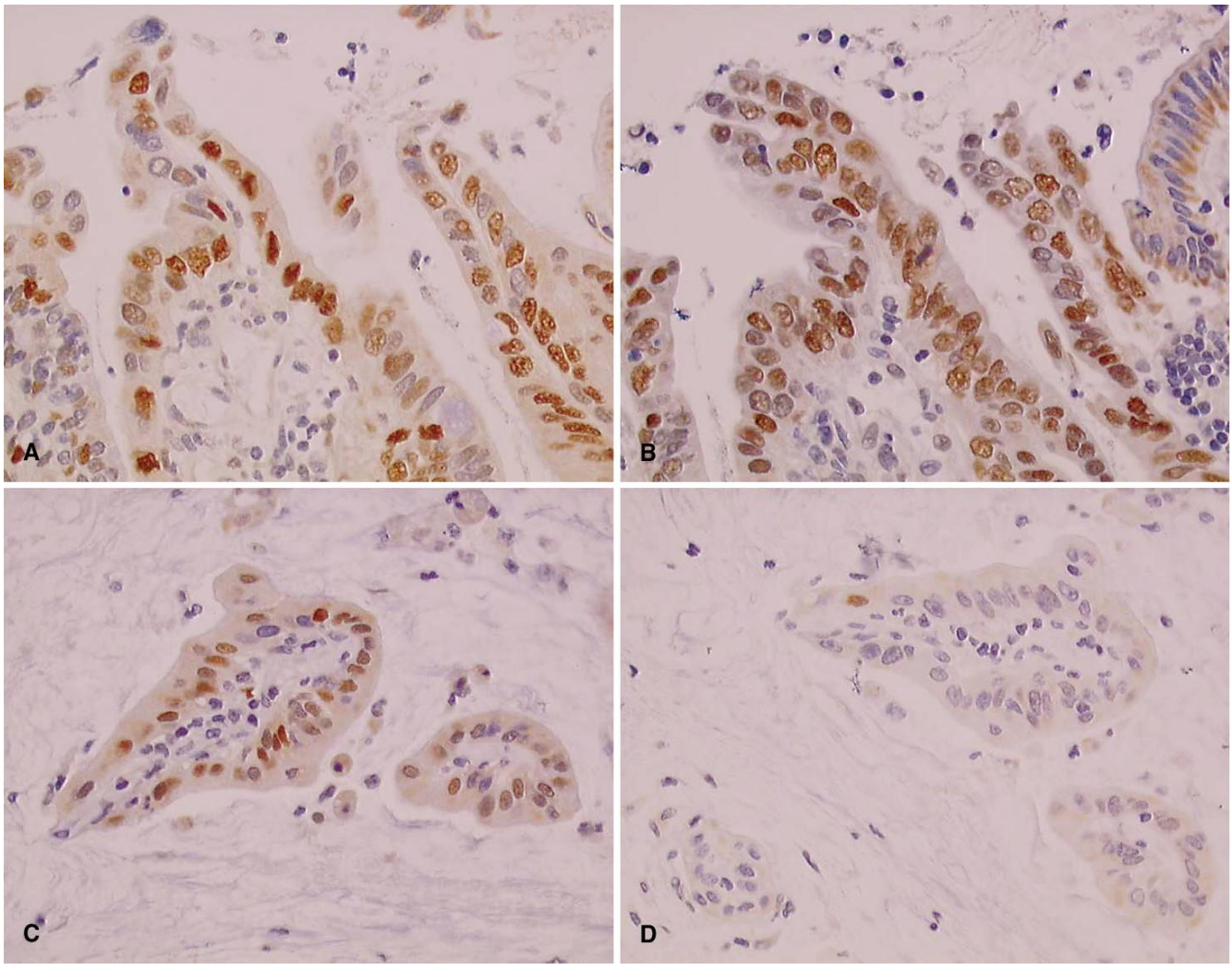

Fig. 1A–D p21 expression in two colorectal adenocarcinomas: one case shows prominent p21 immunoreactivity, which was more diffuse and intense in the superficial areas of the tumour (**A**), with concurrent p53 overexpression (**B**); this tumour showed p53 alteration at PCR-SSCP analysis. The second case shows diffuse p21 immunoreactivity (**C**), while p53 staining was restricted to few isolated nuclei (<0.1%) (**D**); this case did not show p53 alteration on PCR-SSCP analysis. Immunostaining for p21 and p53 with a StrepABC technique with light haematoxylin counterstain

gel loading solution (75% deionised formamide, 12 mM NaOH, 6 mM EDTA, and 0.05% xylene cyanol and bromophenol blue), denatured at 95°C for 5 min, and kept on ice until loading. Electrophoresis was carried out in 10% nondenaturing polyacrylamide gel (29:1 acrylamide to bisacrylamide) containing 5% glycerol at room temperature at 100–150 V for 15–20 h. Electrophoresed DNA was visualised by silver staining of the gel. For all cases we evaluated a tumour sample and a sample of corresponding normal tissue. A band shift was considered to indicate p53 gene mutation. Polymorphisms were excluded by comparing the results obtained on corresponding tumour and normal tissues.

The relations among the variables were evaluated by a Chi-square test and analysis of variance (ANOVA). When multiple relations had to be analysed, we performed a post hoc Sidak's test to determine to which of the relations the *P*-value was referred.

Results

p21 immunoreactivity was evaluable in 98 out of 103 tumours; 5 tumours showing absent p21 immunoreactivity without clear-cut p21 immunoreactivity in internal controls were excluded. Nuclear p21 immunoreactivity was observed in 85 (87%) tumours (Fig. 1) and in the superficial compartment of adjacent normal crypt cells; in 13 tumours no nuclear reactivity for p21 was seen in neoplastic cells. Mean and median percentages of p21 immunoreactive cells were 11% and 4%. Thirty-three cases (39%) with p21 immunoreactivity in 10% or more tumour cells were considered to have high p21 expression. p21 staining was heterogeneous and often detected in clusters of tumour cells; in rare cases p21 staining was more pronounced in superficial areas of tumours. No relation was seen between p21 immunoreactivity and site of the tumours (right vs left), TNM stage or grade (Table 1).

p53 SSCP nonpolymorphic band shifts were identified in 47 (48%) cases (17 in exon 5, 4 in exon 6, 17 in exon 7, 9 in exon 8) and were regarded as an indication of possible p53 gene mutations (Fig. 2). Expression of

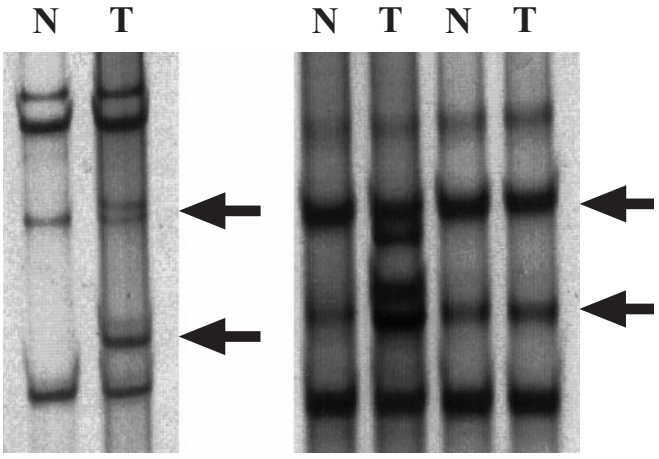

Fig. 2 Nonradioisotopic PCR-SSCP electrophoretic gel with silver staining for exons **A** 5 and **B** 7 of the p53 gene; each tumour sample (*T*) is compared with normal tissue (*N*) of the same patient, and band shifts (*arrows*) are considered as indicative of p53 gene mutation. **B** Results from two different patients, one with a band shift (*left*) and one without (*right*)

p53 protein was identified in 86 cases; mean and median percentage of p53-reacting cells were 40% and 60%, respectively; 60 cases showing p53 expression in 40% or more of tumour cells were considered to overexpress p53 (Fig. 3). p53 SSCP band shift and p53 protein overexpression were strictly associated (Table 2). No relation was seen between p53 alterations at IHC or SSCP level and site of the tumour (right vs left), TNM stage or grade (Table 1).

Expression of p21 was related to p53 status as evaluated with IHC or with SSCP analysis (Table 3), low p21 expression being associated with p53 alterations. The strongest association was seen between low p21 and concurrent p53 overexpression and p53 SSCP band shift (Table 4). We also investigated whether the combined IHC evaluation of p21 and p53 could give more reliable information regarding p53 gene alteration. The relation between the p53/p21 immunophenotype and p53 gene alteration was indeed strictly significant (Table 5). There were, however, some discrepant cases with concurrent high p21 expression and p53 alterations, and conversely, cases with low to absent p21 expression and normal p53.

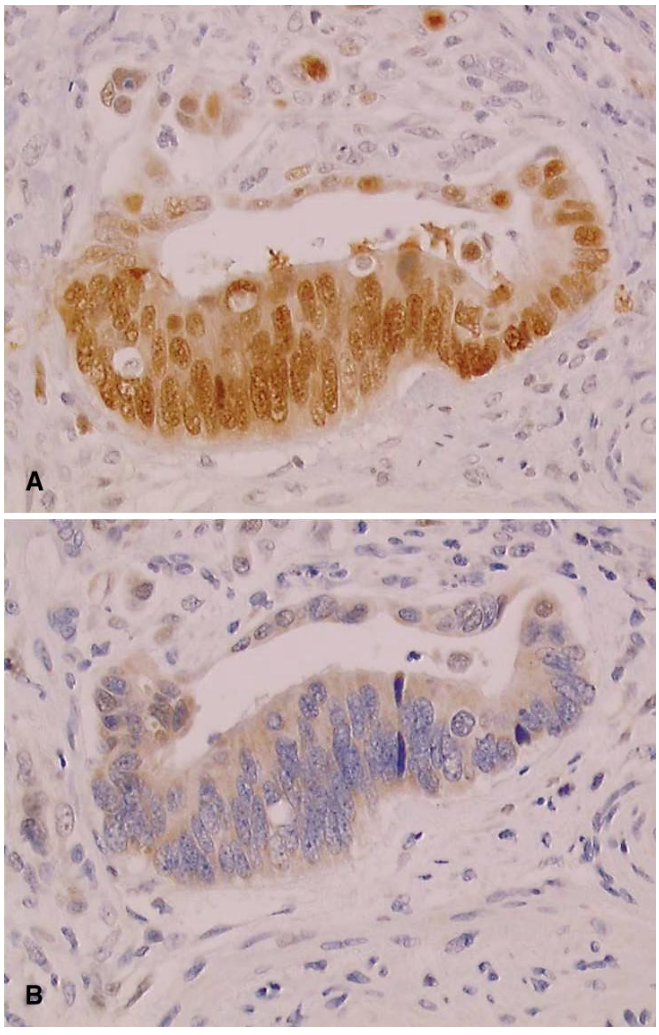

Fig. 3 A Strong p53 expression in colorectal adenocarcinoma. **B** This tumour did not show p21 immunoreactivity and showed p53 alteration on PCR-SSCP analysis. Immunostaining for p53 with a StrepABC technique with light haematoxylin counterstain

Discussion

In this study we were able to confirm previous observations that p21 expression is topographically restricted to nonproliferating and differentiated cells in normal colorectal mucosa and is heterogeneous and diffuse in around one third of sporadic CC [13,15, 35, 37, 40, 41]. We also demonstrated a strong association between alterations to p53 at the IHC and/or PCR-SSCP levels and low p21 expression.

The relation of p21 expression to p53 alterations in human tumours is intriguing: in some tumours, such as lymphomas [44], high-grade astrocytomas [26] and sarcomas [12, 23], p21 seems to be dependent predominantly on p53 induction, while in most others, such as lung [31], laryngeal [34], cutaneous [43], urothelial [10], gastric [39, 45] and oesophageal [39] tumours, p21 expression is independent of the p53 pathway and frequently seems to be related to cell differentiation. Moreover, even for a single tumour type, different studies have shown different results: for breast carcinomas, for example, some studies suggest that p21 expression is related to p53 [5, 17], while others suggest that p21 may be expressed by p53-independent mechanisms [4, 36].

The relation between p53 alterations and p21 expression is not only of academic interest, since, if in a given tumour type it can be demonstrated that p21 expression is dependent upon induction by wild-type p53, it could be suggested that the concurrent evaluation of p21 and p53 alterations could give valuable information on the integrity of the p53 pathway. This in turn could be of

Table 2 Relation between p53 SSCP band shift and p53 overexpression

	p53 IHC ≥40%	p53 IHC <40%	Total
p53 SSCP with band shift	39 (83%)	8 (17%)	47 (46%)
p53 SSCP without band shift	21 (38%)	35 (62%)	56 (54%)
Total	60 (58%)	43 (42%)	103
		P=0.000003[a]	

[a] Chi-square

Table 3 Relation between p21 immunoreactivity and p53 status

	p53 IHC >=40%	p53 IHC <40%	p53 SSCP Shift	p53 SSCP No shift
p21>=10%	15 (44%)	18 (56%)	9 (27%)	24 (73%)
p21<10%	43 (66%)	22 (34%)	37 (57%)	28 (43%)
		P=0.048[a]		*P*=0.005[a]

[a] Chi-square

Table 4 Relation between p53 status as evaluated with IHC and SSCP and p21 expression

	p21>=10%	p21<10%	Total
p53 IHC<40 and SSCP without band shift	16 (50%)	16 (50%)	32 (33%)
p53 IHC≥40 and SSCP without band shift	8 (40%)	12 (60%)	20 (20%)
p53<40 and SSCP with band shift	2 (25%)	6 (75%)	8 (8%)
p53 IHC≥40 and SSCP with band shift	7 (18%)	31 (82%)	38 (39%)
Total	33	65	98
P		0.03[a]	

[a]*P*-value referred to comparison between cases with normal p53 expression and without mutation (row 1) and cases with p53 overexpression and mutated (row 4); variance analysis (ANOVA) followed by post hoc test by Sidak's method

Table 5 Relation between p53 and p21 WAF1 expression and p53 SSCP band shift

	p53 SSCP Band shift	p53 SSCP No band shift	Total
p53 IHC<40 and p21 ^{WAF1}<10	6	16	22
p53 IIC<40 and p21 ^{WAF1}≥10	2	16	18
p53 IHC≥40 and p21 ^{WAF1}<10	31	12	43
P53 IHC≥40 and p21 ^{WAF1}≥10	8	7	15
Total	47	51	98
P	0.00002[a]		

[a] Cases with p53 overexpression and low p21 (row 3) vs those without p53 overexpression with any p21 value (rows 1 and 2); variance analysis (ANOVA) followed by post hoc test by Sidak's method.

clinical value. Theoretical considerations and in vitro studies suggest that integrity of the p53 pathway plays a major part in determining cellular chemo/radiosensitivity [22, 28, 32]. Chemo/radiotherapy induces DNA damage, which activates p53 function, and this in turn blocks the cell cycle to allow DNA repair or apoptosis. However, in vivo studies relating p53 status and chemo/radiosensitivity are not conclusive. In breast carcinomas, for example, conflicting results have been reported: some studies support the hypothesis that tumours without p53 alterations show a larger benefit from therapy [9, 16], while others make any such hypothesis seem weak [1, 38]. In a recent paper we proposed the hypothesis that in breast carcinomas the combined immunohistochemical evaluation of p53 and p21 expression may provide valuable clinical information concerning the response to systemic adjuvant therapy [6].

In the present study we have demonstrated that, in a large series of CC, p21 expression is related to p53. This observation, which is in keeping with the findings of some previous studies [13, 15, 37, 40], suggests that in CC, unlike most other human neoplasms, p21 expression is mainly dependent on p53 induction and may probably be considered as a marker of integrity of the p53 pathway. Although not universally accepted [41], this hypothesis suggests that further studies on CC, relating the alterations of p53 and p21 to clinical outcome of the patients, could be of interest. Investigation of the clinical response of CC to radio/chemotherapy in relation to p53 and p21 alterations could be even more interesting, since it has been suggested that in CC mutated p53 increases sensitivity to radiation and drugs [25].

The above relation between p21 expression and p53 function in CC shows some exceptions. In our series we detected 17 cases with high p21 expression in spite of p53 alterations, and in 8 of these there were concurrent IHC and PCR-SSCP p53 alterations. We might speculate that in these cases mutated p53 is still able to activate the expression of the p21/WAF1 gene [29] or that high p21 expression is related to posttranscriptional mechanisms

[19, 27]. Alternatively, it must be borne in mind that p21 induction is indeed multifactorial (reviewed in [11]) and that p53, although important, is not the only inducer of p21 expression in CC [11, 42]. The hypothesis has indeed been put forward that in normal colonic epithelia cell–cell or cell–stroma interactions may contribute to cell-cycle control influencing the replicative machinery resulting in induction of p21 in a topographically restricted pattern [15]: this regulation is progressively lost in the adenoma–carcinoma sequence in CC [13, 35], but it is reasonable to suppose that in some CC it might be at least partially conserved. In a previous study on CC we did indeed observe that in a few cases there was a sort of compartmentalisation of p21 expression in superficial areas of the tumours or in areas of more pronounced glandular differentiation [13]. In the present study we confirmed this observation, which is in keeping with the hypothesis that in some CC p21 expression could still be under the control of mechanisms related to topographical/differentiation patterns [15].

In 16 of our cases, we detected low p21 expression in spite of "normal" p53 at the IHC and PCR-SSCP levels. These cases could represent situations in which p53 is indeed altered but neither IHC-based nor PCR-SSCP-based analyses can detect these alterations. Alternatively, these cases could reflect situations in which normal p53 is unable to induce high expression of p21 because of alterations of the p21 itself (which are known to be very rare). A further hypothesis concerns alterations in the p21 post-transcriptional metabolism, such as degradation pathways.

In the present series of cases p53 alterations at the IHC or PCR-SSCP level were seen in 58% and 46% of CC respectively; when the combined data from IHC and PCR-SSCP analyses were considered, p53 alterations were seen in 67% of cases. These data are in keeping with the large series of data in the literature concerning p53 alterations in CC, and further underscore the fact that each of these analyses may underestimate the real frequency of p53 alterations, emphasising that each should be considered complementary to the other.

In conclusion, our study shows that p21 expression in CC is mainly related to p53 functional status, supporting the hypothesis that p21 expression could be an interesting adjunct in the evaluation of the functional status of the p53 pathway in CC. Moreover, our data suggest that further studies coupling p53 and p21 analysis in relation to clinical outcome and possibly therapy response in CC could provide clinically relevant information.

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