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Original Paper

Prognostic Significance of Cytoplasmic p53 Overexpression in Colorectal Cancer. An Immunohistochemical Analysis

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p53 overexpression was studied by immunohistochemistry in 96 consecutive colorectal cancer patients, subdividing positive specimens according to two staining patterns: cytoplasmic or nuclear. Forty-seven per cent of the cases were p53 positive, a significant correlation being found with Dukes' stage ($P = 0.0036$). A prevalence of nuclear staining was observed in Dukes' B and cytoplasmic in Dukes' D stages. After 36 months, 23% of the patients had a recurrence, and 45% were p53 positive, all Dukes' C–D stage with cytoplasmic staining. The Kaplan–Meier curve showed a significant correlation between p53 cytoplasmic staining and disease-free survival period ($P = 0.002$). With respect to disease-free survival, the Cox proportional hazard regression test, comparing p53 positivity with Dukes' stage, showed the latter to be the most significant variable. In our series of patients, advanced Dukes' stage tumours were localised in the right colon, where a higher percentage of p53 positivity (67% versus 40% of the left side), as well as a higher frequency of cytoplasmic staining was observed. In conclusion, from the data obtained, a strong correlation between p53 cytoplasmic staining and patient prognosis is clearly indicated.

Key words: p53 protein, cytoplasm, colorectal cancer, prognosis, immunohistochemistry
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INTRODUCTION

COLORECTAL CANCER is the second leading cause of cancer death in Western countries, and surgical excision is the best treatment option. Moreover, approximately 40% of patients undergoing curative resection of their primary tumour will have local, regional or distant recurrences. Except for Dukes' staging, there are few pathological parameters correlating with tumour aggressiveness, thus it is of critical importance to identify specific biological markers which accurately predict tumour progression and recurrence.

Colon carcinogenesis is believed to be the result of genetic alterations to specific genes, resulting in activation of proto-oncogenes and/or inactivation of tumour suppressor genes which leads to malignant conversion of adenomas to carcinomas. In the adenoma–carcinoma sequence proposed by Vogelstein and colleagues [1], the colorectal multistep tumorigenesis is characterised by abnormalities of oncogenes and oncosuppressor genes, such as *APC* (Adenomatous Polyposis Coli) [2], *RAS* [3] *DCC* (Deleted in Colorectal Cancer) [4], *MCC* (Mutated in Colorectal Cancer) [5] and *TP53*, the latter being associated with the development of invasive carcinoma [6].

TP53 is the most frequent altered oncosuppressor gene in cancer, and its overexpressed product, a 53-Kd phosphoprotein, is found in all types of human tumours [7]. p53 accumulation, due to a lowered turnover, is most frequently the result of gene mutation on one allele, accompanied by deletion of the other allele, which inhibits, at the nuclear level, the protein which controls the critical step of cell cycle progression at the G1/S transition [8]. The loss of the fundamental *TP53* "guardian" role in cell proliferation, along with other gene mutations, results in an irreversible neoplastic progression [9].

Evidence has also been obtained of cytoplasmic p53 gene product localisation, not necessarily ascribed to gene mutations and detectable in different proliferative situations [10–14]. Cytoplasmic localisation, also involving a functional p53 inactivation, may be due to binding to viral or cellular products [15,16], may be consequent to inefficient nuclear translocation [14] and may depend on the conformational and phosphorylation status of the protein [17–19].

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Among human cancers, cytoplasmic p53 overexpression patterns have been detected in glioblastomas [20], neuroblastomas [14], malignant melanomas [21], lymphomas [22], prostate carcinomas [23], breast [13] and colorectal tumours [24,25]. As for colorectal cancer, recently published data clearly indicate that worse prognosis is related to p53 cytoplasmic accumulation [26].

This immunochemical study examined a group of 96 consecutive colorectal cancer patients, who were followed for at least 3 years after surgery. Our aim was to compare tumour p53 "cytoplasmic" and nuclear overexpression patterns with patients outcome, in terms of disease-free survival, in order to assess the usefulness of "cytoplasmic" p53 overexpression pattern as a prognostic factor. We also analysed left and right-side colon tumour distribution with respect to p53 immunostaining, in order to evaluate any differential overexpression pattern and determined whether a p53 positivity could be detected at a distance from the tumour in view of possible local recurrences.

PATIENTS AND METHODS

Patients

The present study included 96 consecutive cases of colorectal cancer who underwent curative resection at the Clinical Surgery Institute of the Catholic University School of Medicine, Rome, from 1991 to 1994. No patient received either pre-operative or postoperative chemotherapy or radiotherapy.

The patients, 57 males and 39 females, were classified according to Dukes, with 2 stage A, 56 stage B, 27 stage C and 11 stage D. Stage D patients underwent curative resection of liver metastases. There were five well differentiated, 60 moderately and 31 poorly differentiated carcinomas. Table 1 shows patients grouped according to tumour localisation and subdivided by staging and grading. The mean age was 63 years, range 28–85. Follow-up data were available for all patients. The initial evaluation was made 36 months after surgery (range 12–36). During that period 22 patients had a recurrence and 16 of these died from colorectal cancer.

Patients specimens obtained after surgical resection were snap frozen and maintained at –80°C until examination.

For some of the patients, biopsies were taken up to 9 cm proximal and distal to the resection site (1 sample/cm), which was histologically free from tumour. In 8 patients from this group, randomly selected, proximal and distal samples were examined after verifying tumour p53 positivity.

Immunostaining

For our study two antibodies were used: (1) the anti-p53 polyclonal CM-1 [27] and (2) the DO-7 monoclonal antibody, the latter able to detect both wild-type and mutated forms of the oncosuppressor *TP53* gene product [28]. Cryostat 5μ sections, acetone fixed for 15 min, were treated to block edogenous peroxidase with 0.3% H_2O_2 in methanol for 30 min. After blocking with 1% bovine serum albumin (BSA) for 1 h, sections were incubated with primary antibodies for 2 h at room temperature, followed by biotinylated antiserum for 30 min. The CM-1 antibody was utilised at a dilution of 1:700 and the DO-7 antibody at a dilution of 1:1400 in phosphate-buffered saline (PBS). The avidin–biotin complex was then applied for 30 min and the reaction was visualised with diaminobenzidine (DAB) (Vectastain ABC kit, Vector Labs). The sections were lightly counterstained with Harris' haematoxylin. Positive controls were performed using a p53 positive colon carcinoma, and negative controls were duplicate sections using an unrelated antibody and PBS.

We evaluated the significance of p53 overexpression frequency difference distinguishing nuclear and cytoplasmic (whether or not with associated nuclear) positivity patterns. The samples were scored by three independent pathologists without any pathological or clinical information concerning the cases under study. p53 was considered positive when tumour cells were stained irrespective of positivity percentage. Furthermore, staining intensity varied greatly even in the same microscopic area. In some cases, p53 staining was more pronounced at the infiltrative margins of the tumours.

Statistical analysis

The correlation between p53 positivity and the established clinicopathological parameters was tested by the Pearson χ^2 test.

Disease-free survival was evaluated after the Kaplan–Meier method, and the obtained curves compared by the log-rank test.

The Cox proportional hazard regression model was used to compare the prognostic value of p53 overexpression and of Dukes' stage with respect to survival.

RESULTS

Immunohistochemical evaluation was made with p53 positivity defined as nuclear (Figure 1a), or cytoplasmic (with or without nuclear staining) patterns (Figures 1b and 1c), as detected with the CM-1 polyclonal and the DO-7 monoclonal antibodies. In comparing tissue immunoreactivity by CM-1 and DO-7, all the samples showed the same results with both antibodies.

Forty-five of the 96 tumour specimens (47%) had p53 positivity, 27 (28%) with nuclear and 18 (19%) with cytoplas-

Table 1. Colorectal cancer patients according to tumour localisaton stage and grade

Localisaton	*n*	Stage A	Stage B	Stage C	Stage D	Well differentiated	Moderately differentiated	Poorly differentiated
Caecum	6	0	1	1	4	0	2	4
Ascending colon	13	0	5	8	0	0	8	5
Tranverse colon	5	0	5	0	0	0	1	4
Total right	24	0	11	9	4	0	11	13
Decending colon	10	1	4	4	1	0	6	4
Sigmoid colon	25	0	17	4	4	2	20	3
Rectum	37	1	24	10	2	3	23	11
Total left	72	2	45	18	7	5	49	18
Total	96	2	56	27	11	5	60	21

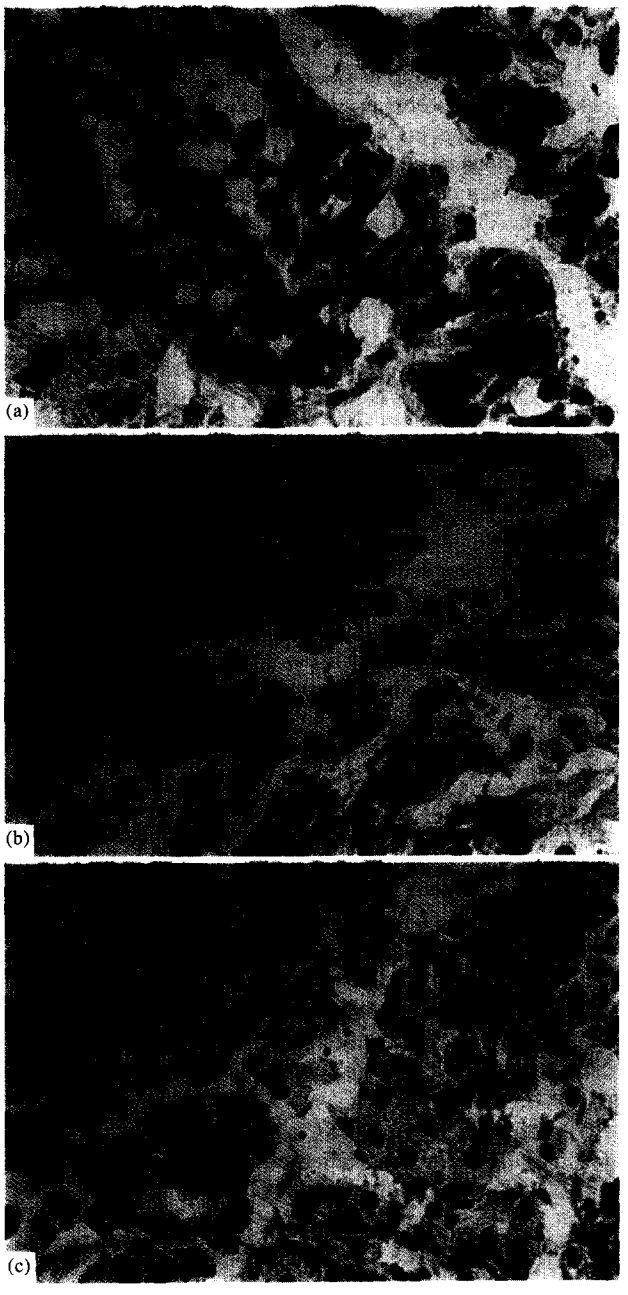

Figure 1. p53 overexpression pattern in colon carcinoma. (a) nuclear; (b) nucleocytoplasmic and (c) cytoplasmic. Magnification × 200.

mic. The total p53 positive cases were analysed in relation to the established clinicopathological parameters, that is, age, sex, grade and Dukes' stage. In univariate analysis, no correlation was found with patients sex, age and tumour grade. A postive correlation was observed with Dukes' stage ($P = 0.0036$).

Regarding p53 staining patterns, Table 2 indicates that there was a prevalence of nuclear staining in Dukes' B and cytoplasmic postivity in Dukes' D stages.

After 36 months, an evaluation of recurrences was performed, an initial prediction of disease-free survival being confirmed by Dukes' stage ($P < 0.00009$) (Figure 2). 22 of the 96 patients (23%) had a recurrence and 16 of these died from colorectal cancer. Recurrences occurred in 5 patients with

Table 2. Distribution of p53 positivity patterns with respect to Dukes' stage

Staining pattern	Dukes' stage A	B	C	D
Nuclear	0	22	5	0
Nucleocytoplasmic and cytoplasmic	0	5	6	7
Negative	2	29	16	4

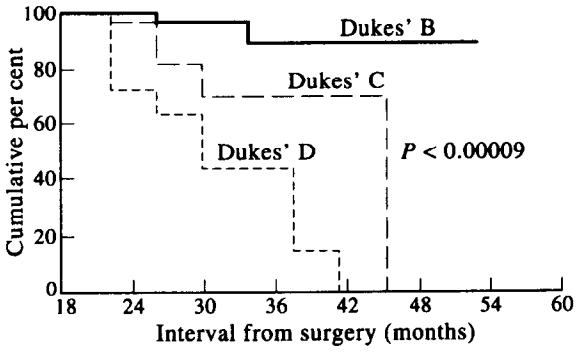

Figure 2. Evaluation of recurrences after 36 months of follow-up in the 96 colorectal cancer patients under study. A significant correlation between survival and Dukes' stage was observed.

Dukes' B, 8 with Dukes' C and 9 with Dukes' D stage. Among the 22 cases with recurrence, 10 patients (45%) were p53 positive. All the positive cases had cytoplasmic staining and were Dukes' C or D stage.

Figure 3 shows the Kaplan–Meier disease-free survival curve for 96 patients, showing that cytoplasmic p53 expression was associated with shorter survival ($P = 0.002$).

A Cox proportional hazard regression model was also calculated with respect to disease-free survival, in order to evaluate the prognostic significance of p53 overexpression in relation to Dukes' stage. The obtained data show that the latter is the most significant variable in terms of prognosis ($P = 0.0002$).

Among the 96 patients under study, the total right-sided tumours showed a higher frequency of advanced Dukes' stages (C–D) than the total left-sided ones, where a prevalence of B stage tumours could be observed (see Table 1). Regarding p53 positivity, a higher percentage of positive tumours was

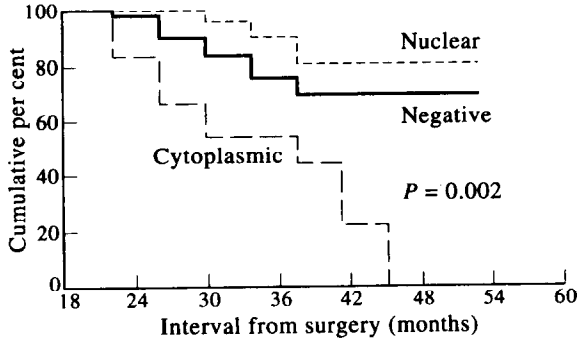

Figure 3. Evaluation of p53 overexpression patterns distribution after 36 months of follow-up in the 96 colorectal cancer patients under study. A significant correlation is shown between disease-free survival and the "cytoplasmic" pattern.

localised in the right colon segment, with 16 of 24 right-sided (67%) and 29 of 72 left-sided tumours (40%) positively stained. In relation to left/right localisation and Dukes' stage, the p53 positivity patterns were differently distributed. A higher frequency of cytoplasmic positivity (9/16 positive cases, 56%) was detected in the right-sided carcinomas compared with the left-sided tumours (9/29 positive cases, 31%). Nuclear positivity was more frequent in left-sided tumours (20/29 positive cases, 69%) compared with the right-sided ones (7/16 positive cases, 44%) (Figure 4). No statistical significance was reached between tumour localisation and p53 positivity patterns. As for p53 positivity distribution in left/right side recurrences, 6 of the total 10 positive cases were in the right colon and all had cytoplasmic staining.

In our study, we also analysed p53 overexpression in biopsy specimens taken up to 9 cm proximal and distal to the resection site. 6 of the 8 randomly selected p53 tumour positive cases were positive at distance, p53 staining being observed not further than 3 cm distally and/or proximally. Staining, exclusively nuclear, was restricted to transitional mucosa adjacent to cancer localisation.

DISCUSSION

p53 overexpression in colorectal cancer, associated with other gene alterations, seems to be related to patient outcome, as demonstrated by a large number of studies. Its significance as an independent prognostic factor is not clear-cut, although, as stated, its appearance in the malignancy is clinically valuable. Its determination, at least by immunohistochemistry, may become a valuable tool for both the diagnosis and prognosis of colorectal as well as other types of human cancer [29].

In this study, we analysed a series of 96 consecutive colorectal cancer patients, treated exclusively by surgery, 11 presenting with liver metastases (Dukes' D stage). p53 positive immunostaining was detectd in 47% of cases, in agreement with other studies [30,31]. The univariate analysis, comparing p53 positivity with the established clinicopathological parameters, indicated Dukes' stage was significantly related to p53 overexpression, thus confirming other authors observations [24].

In performing p53 analysis, we related patients clinicopathological variables with p53 immunostaining patterns, differentiated into "cytoplasmic" and nuclear. The two patterns represent differential inactivation modalities, and it has been demonstrated in colorectal cancer that cytoplasmic positivity significantly relates to patient prognosis [26]. In our study we distinguished the "cytoplasmic", including the nucleocytoplasmic staining from nuclear staining, in order to verify whether such a discrimination would yield a more significant correlation with clinicopathological parameters, especially Dukes' stage, and with disease-free survival.

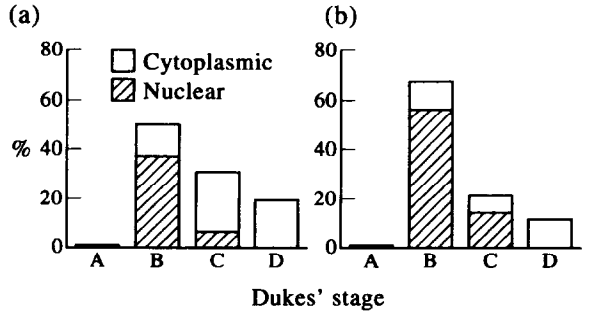

Figure 4. Differential p53 overexpression pattern between right (a) and left (b) hemicolic side. A prevalence of cytoplasmic staining was detected in the right colon in relation to Dukes' stage.

In our series of patients only cytoplasmic staining was seen in Dukes' D stage, while nuclear staining was more frequently observed in Dukes' B stage tumours. During the 36 months follow-up period, 22 patients had a recurrence and 45% were p53 positive, all Dukes' stage C–D tumours with cytoplasmic positivity. This indicates that p53 cytoplasmic staining is related to a poorer prognosis. The Kaplan–Meier curve confirmed a significant difference ($P = 0.002$) between the two patterns. However, when applying the Cox proportional hazard regression model with respect to disease-free survival in order to relate Dukes' stage with p53 staining, the former was the more significant variable. From our data, on a 36 months follow-up period, it can be concluded that a more accurate indication of patient prognosis can be drawn from the p53 staining pattern. Nevertheless, according to Sun and associates [25], with a longer follow-up period, nuclear p53 localisation seems to acquire a decisive value in terms of prognosis.

In our group of patients, where Dukes' D were more represented than Dukes' A stages, we found a high frequency of advanced stage tumours in the right side of the colon (see Table 1) and more p53 positivity (67% versus 40% in the left side). Consequently, when analysing p53 distribution in the right and left colon side (see Figure 4), we observed a prevailing cytoplasmic immunostaining in the right colon. Although a statistically significant difference was not reached, the observed data are in agreement with those from other authors, showing a more frequent cytoplasmic p53 localisation in right side tumours [25].

Finally, in 8 randomly selected p53 positive patients, we examined 9 cm colon proximal and distal regions to the resection site to verify whether any p53 positivity could be detected in the adjacent transitional mucosa. We observed p53 positivity, exclusively nuclear, in 6 cases, spanning not further than 3 cm proximal or distal to the tumour site. This suggests that, in p53 positive cells eventually constituting a neoplastic focus, the "cytoplasmic" pattern might hypothetically follow an initial nuclear localisation.

In conclusion, from our data, a strict correlation between cytoplasmic p53 immunohistochemical pattern and patient prognosis in colorectal cancer is shown, confirming observations from other authors that cytoplasmic p53 localisation should be considered a valuable prognostic indicator [24–26]. However, these data need to be strengthened by increasing the number of patients and, importantly, extending the follow-up period. However, the immunohistochemical observations will be better understood when the differential p53 localisations in tumour tissue are unravelled by molecular biology approaches.

As for p53 overexpression distribution between right and left colon side, the series of patients, consecutively collected, that we studied has shown a higher frequency of advanced stage tumours in the right hemicolic side. Thus, it may be hypothesised that the observed p53 staining patterns are, as a consequence, related to tumour progression and aggressiveness. Whether true oncogenetic differences between right and left colon exist, bringing about differential p53 overexpression behaviour, is still not known.

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