ORIGINAL PAPERS

Correlations of Proliferation Markers, p53 Expression and Histological Findings in Colorectal Carcinoma

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Abstract

Aim. To investigate the expression of PCNA, Ki-67 and p53 antibodies in colorectal carcinomas and to establish the relationship between these markers and some particular histological findings of colorectal carcinomas. **Material and methods.** We determined immunohistochemically the expression of PCNA, Ki-67 and p53 antibodies in 41 cases of colorectal carcinomas. **Results.** In adenocarcinomas, the tumor proliferative activity, detected with PCNA and Ki-67 antibodies, increased with the histological grade. Mucinous adenocarcinomas had a mean PCNA LI of 50% and a mean Ki-67 LI of 32%, while signet ring carcinomas had a mean PCNA LI of 70% and a mean Ki-67 LI of 45%. The proliferative activity in the foci of squamous metaplasia was lower than the proliferative activity of malignant areas in the analyzed adenocarcinomas. The p53 overexpression was detected in 24 cases (58.53%). In adenocarcinomas, the p53 positive rate increased with the dedifferentiation of these tumours. Only 16.66% of the cases of carcinomas with mucus secreting cells overexpressed p53, while adenocarcinomas overexpressed this protein in many more cases (65.71% of the cases). The overexpression of p53 was associated with the highest PCNA and Ki-67 LI. **Conclusions.** The foci of squamous metaplasia, present in colorectal adenocarcinomas, do not seem to influence the increase of the tumours. The p53 overexpression was associated with nonmucinos colorectal carcinomas and with the histological grade of colorectal adenocarcinomas. The p53 over expression tended to be more frequent in colorectal carcinomas with high proliferative activity.

Key words

Colorectal carcinoma - PCNA - Ki-67 - p 53 - immunohistochemistry

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Introduction

Antibodies that recognize nuclear proteins associated with tumour cell proliferation can be determined by immunohistochemistry and represent an attractive alternative to the analysis of cell proliferation by flow cytometry. Currently, PCNA and Ki-67, and its epitope MIB-1, are the most popular antibodies investigated as prognostic factors in colorectal carcinoma.

Proliferating cell nuclear antigen (PCNA) is a 36-kd DNA polymerase delta auxiliary protein that complexes with cyclin D and cyclin-dependent kinases. It is involved in the proliferation of neoplastic as well as non-neoplastic cells and it is specifically expressed in proliferating cell nuclei (1). This specific antibody recognizes PCNA protein, which is at the maximum level in the late G1 and S phase of proliferating cells (2).

The Ki-67 antigen is a non-histonic protein useful for identifying the proliferative cells (3). Though the flow cytometry methods showed that Ki-67 is expressed in all the cell cycle phases except G_0 phase, its detection by immunohistochemical methods is limited to its appearance in the late G_1 phase with maximum expression in prophasis and metaphases. Then its immunoexpression diminishes in anaphases and telophasis, its levels being apparently undetectable during most of the interphase (4). For these reasons, Ki-67 is recognized as a mitotic activity indicator. A rise in Ki-67 expression indicates a rise of the mitotic activity and of the cell proliferation (5). The use of the monoclonal antibody MIB-1 allows effective staining of Ki-67 antigen in routine formalin-fixed, paraffin-embedded tissues. Proliferative activity can be quantitatively and comparatively assessed when the percentage of positive stained cells is calculated (6). Though presently there are many antibodies that can be used on paraffin-embedded sections (MM1, NCL-ki-67p, Rah Ki-67, MIB 1), many studies have shown that MIB-1 antibody has the highest sensitivity and offers the best visual staining. MIB-1 is also very useful compared to other antibodies when the labelling index (Ki-67 LI) is used as a criterion for tumour grading or for prognostic evaluation (7).

The p53 protein is a DNA-binding cell cycle-regulating transcription factor that sits at the crossroad of pathways governing orderly cell division and the balance between cell survival and cell death. On the cellular level, losses, deletions, and mutations of the P53 play a crucial role in the pathogenesis of a strikingly large number of malignancies, including colorectal cancer (8).

There is no doubt about the role of p53 mutations in the progression of colorectal tumours. The p53 protein is important in maintaining DNA integrity. DNA damage results in p53 mediated arrest in the G1 phase of the cell cycle, followed by repair or, if the damage is too great, p53 induced apoptosis. Therefore, loss of function of p53 by mutation or deletion allows cells to accumulate mutations throughout the genome and results in karyotypic instability, impaired G1 cell cycle arrest, and reduced apoptosis (9).

The aim of this study is to investigate the relationship between the cell proliferative activity, detected with PCNA and Ki-67 antibodies, and the expression of p53 in colorectal carcinoma, as well as between these markers and some particular histological findings (the histological type, the grade of tumours and the association with squamous metaplasia). Although previous papers clarified the role of these markers in colorectal malignancy, there is little data in our geographical region. Furthermore, this is one of the first objectives of a three year project that will investigate molecular markers in colorectal malignancy arising within the south–west of Romania, by using state-of-the-art molecular diagnosis techniques.

Material and methods

Patients

Tumour tissues from 41 cases of colorectal carcinoma (23 male, 18 female patients; mean age 60 years) were investigated. All patients had been admitted to the Emergency County Clinical Hospital of Craiova in 2005. Tumour tissues were initially processed through the classical method (formalin fixed and paraffin embedded). The studied tumours were classified according to WHO histological classification (10) : adenocarcinomas (grade 1, well differentiated - 5 cases; grade 2, moderately differentiated - 21 cases and grade 3, poorly differentiated - 9 cases), mucinous adenocarcinomas (4 cases) and signet ring cell carcinomas (2 cases). Five cases of adenocarcinomas (one case of grade 1 and 3 cases of grade 2) presented small foci of squamous metaplasia. The squamous component represented less than 10% of the tumour cells.

Immunohistochemistry

Immunostaining for PCNA, Ki-67 and p 53 was performed on 4-µm thick tissue sections onto Superfrost glass slides and dried overnight at 56°C. Sections were deparaffinized in xylene and rehydrated with alcohol. Endogenous peroxidase activity was inhibited by incubating the slides in 3% hydrogen peroxide for 10 minutes, followed by a 5-minute-rinse in distilled water. For the antigen retrieval, the sections were immersed in 10mM citrate buffer (pH 6.0), heated in a domestic microwave oven (750W) for 5x3 min and cooled in running water. The sections were washed in phosphate buffer saline (PBS), and then subjected to blocking of non-specific reactions in normal pig serum. The tissues were incubated with primary antibodies: PCNA (clone PC 10, DAKOCytomation Denmark, diluted 1:100), Ki-67 (clone MIB-1, DAKOCytomation Denmark, diluted 1:10) and p53 (clone DO 7, DAKOCytomation Denmark, diluted 1:50) for 30 min at room temperature. After rinsing in PBS, we applied the streptavidin-biotin technique using LSAB 2 DAKO kit, according to manufacturers' instructions. Positive staining was visualized with 3.3'-diaminobenzidine substrate solution. The sections were counterstained with Mayer's haematoxylin.

Negative external control sections for each case were treated identically except that the primary antibody was replaced with phosphate buffer saline.

Positive external control sections containing tissue from a tonsil (for PCNA and Ki-67), and from an invasive adenocarcinoma sample known to be positive for p53, were included in each staining run. In addition, infiltrating lymphocytes in the tumour stroma served as internal positive control for Ki-67.

Assessment of PCNA and Ki-67

The PCNA and MIB-1 immunostained sections were light-microscopically evaluated using a total magnification of 400x and a 10x10 square grid placed in the ocular. 6-9 sites within the tumour were examined, excepting the areas with tissue enfolding, necrosis and hemorrhagic infiltrate. Nuclear staining stronger than the non-specific reaction of the background tissue was regarded as positive. Cytoplasmatic staining was considered as artifact. The PCNA and MIB-1 labelling indexes (LI) were determined by counting more than 1000 tumour cells, and were calculated as the percentage of positive labelled nuclei.

Assessment of p53

p53 immunostaining was assessed using a light microscope and a visual grading system based on the number of positively stained nuclei of the malignant cells in each section. If 5% or more of the malignant nuclei were stained, the slide was scored as positive. If fewer than 5% of the nuclei were stained, the slide was scored as negative. The staining distribution for p53 was determined for each slide. The staining distribution was either focal or diffuse. The samples with 5-50% stained malignant cells were considered focal, and those with more than 50% stained malignant cells were scored as diffuse.

Statistical analysis

Differences between the patient subgroups were performed by the two-sample t-test (two independent samples). Since this parametric method makes assumptions about normality and similar variances, both the Kolmogorov-Smirnov and Shapiro-Wilk W normality tests were performed. The equality of variances assumption was verified with the Levene's test using F-Fisher statistics. In the case of the two-sample t-test, the non-parametric alternative given by

the Mann-Whitney U test was used, since in some instances it may even offer greater power to reject the null hypothesis than the t-test. The one-way analysis of variance (ANOVA) method was used in order to look at all the data simultaneously. A p-value less than 0.05 was considered statistically significant. All statistical calculations were performed with Statistica for Windows v. 6.0 (Statsoft).

Results

PCNA

Positive nuclear immunohistochemical staining for PCNA (clone PC 10) was seen in all the 41 colorectal carcinomas that were examined. Staining within the tumour nuclei was diffuse (predominantly) or granular, with a higher intensity at the nucleolus. Cytoplasmatic staining was not observed. In normal mucosa, we observed PCNA labeled cells located in the colonic crypts (Fig.1). The stained cells had a uniform arrangement in the lower half of the crypts (the active proliferative zone).

In contrast to the adjacent normal colon mucosa, tumour cells revealed a heterogenic PCNA expression. Thus, tumoral areas with a more intense proliferative activity, identified with PCNA, alternated with areas where the tumoral cells stained with PCNA were fewer. In addition, in adenocarcinomas, in the same malignant glandular structure, we also found a heterogenic PCNA expression. The PCNA index of the investigated tumours ranged from 20 to 97% with a mean PCNA index of 60%.

In well differentiated adenocarcinomas, the mean PCNA index was 27% (ranges 20-30%); in moderately differentiated adenocarcinomas the mean PCNA index was 53% (ranges 25-74%) and in poorly differentiated adenocarcinomas the mean PCNA index was 72% (ranges 43-97%). Therefore, regarding the histological grade of adenocarcinomas, the tumor proliferative activity increased with the decrease in the degree of cell differentiation and was increased above normal mucosa. The differences between grade 1, 2 and 3 adenocarcinomas were statistically significant ($P<0.05$).

In the specimens of colorectal adenocarcinoma which presented small areas of squamous metaplasia, we observed a low number of PCNA positive cells in areas of squamous metaplasia compared with a high number of PCNA positive cells at the level of the carcinomatous glandular structures (Fig.2).

In the cases with microscopical features of mucinous carcinoma and signet ring carcinoma, the mean values of the PCNA-labelling index were 50% (with ranges of 23-71%), and, respectively, 70% (65% and respectively, 75%). These values were similar to moderately and, respectively, poorly differentiated adenocarcinomas.

KI-67 (MIB 1)

Positive nuclear immunohistochemical staining for Ki-67 (MIB-1) antibody was seen in all 41 colorectal carcinomas. One case of mucinous adenocarcinoma showed an associated cytoplasmatic staining which was considered an artifact and the specimen was excluded from interpretation.

Similarly to PCNA, MIB-1 immunostaining within the tumour nuclei was diffuse or granular, but the epithelial tumoral cells were easily identified, especially cells in mitoses. Strong nuclear positive staining was seen in infiltrating lymphocytes within the tumour stroma and acted as internal positive controls. In normal adjacent mucosa, Ki-67 labelled cells were observed in the colonic crypts. These normal epithelial Ki-67 labeled cells were also found, uniformly arranged, in the lower halves of the crypts in all cases.

All tumors showed a heterogenic MIB-1 expression. Thus, in the same tumour areas were present with intense proliferative activity in the vicinity of areas with low proliferation. Similarly to PCNA immunostaining, in adenocarcinomas, Ki-67 immunostaining presented a marked heterogeneity in the same malignant glandular structure, but the heterogeneity for Ki-67 staining was more obvious than the heterogeneity for PCNA staining. The Ki-67 index of the investigated tumours ranged from 14% to 87% with a mean Ki-67 index of 48%.

The mean Ki-67 LI increased with the histological grade of adenocarcinomas and had the following values: 20% in well differentiated adenocarcinoma (ranges 14-23%), 34% in moderately (ranges 18-57%), and 57% in poorly differentiated adenocarcinoma (ranges 35-87%) (Fig.3). The difference between grade 1 and grade 2 adenocarcinomas was not significant, while the difference between grade 2 and 3 adenocarcinomas was significant ($p<0.05$). We observed a low number of Ki-67 positive cells in areas of squamous metaplasia compared with a high number of Ki-67 positive cells at the level of the carcinomatous glandular structures.

Table I PCNA and Ki-67 in histological types

Histological type	Mean PCNA LI (%)	Mean Ki-67 LI (%)
Adenocarcinoma		
grade1	27 *	20 **
grade 2	53 *	34 *
grade 3	72	57
Mucinous adeno-carcinoma	50	32
Signet ring carcinoma	70	45

*p<0.05, ** p not significant

The carcinomas with mucus secreting cells had a 32% (17-55%) mean Ki-67 LI for mucinous adenocarcinomas (Fig.4) and, respectively, 45% (44%, respectively, 46%). for signet ring adenocarcinomas. The mean Ki-67 LI for mucinous adenocarcinomas was similar to the mean Ki-67 LI for moderately differentiated adenocarcinomas, but the mean Ki-67 LI for signet ring carcinomas was lower than that for the poorly differentiated adenocarcinomas.

The mean Ki-67 LI was lower than the mean PCNA LI in adenocarcinomas with the same histological grade or in tumours with the same histological type.

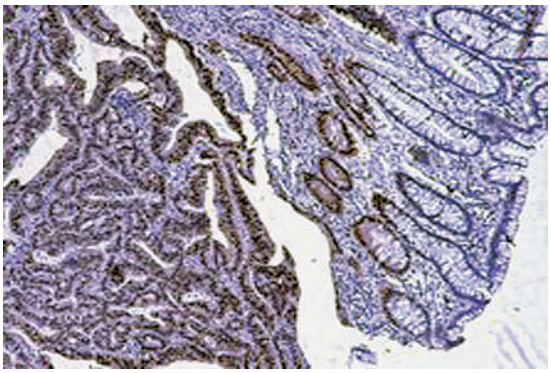

Fig.1 PCNA staining in G2 colorectal adenocarcinoma and in the lower half of adjacent normal colonic crypts, x40.

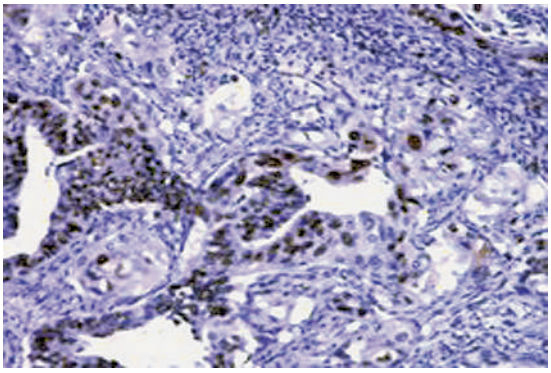

Fig.2 PCNA staining in G2 colorectal adenocarcinoma with foci of squamous metaplasia, x100.

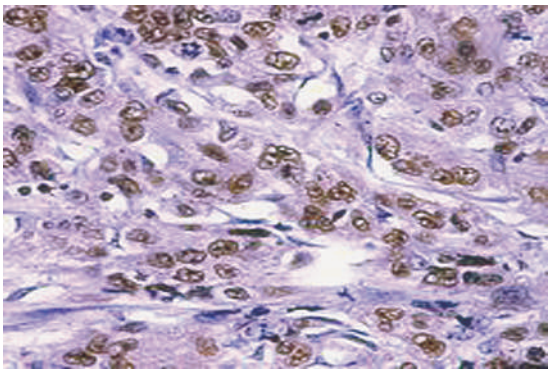

Fig.3 Ki-67 staining in G3 colorectal adenocarcinoma, x200.

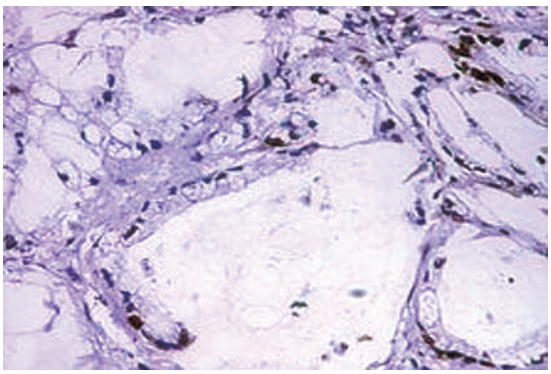

Fig.4 Ki-67 staining in mucinous colorectal adenocarcinoma, x100.

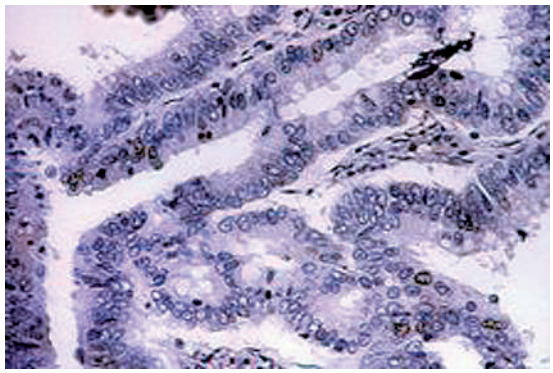

Fig.5 p53 staining in G2 colorectal adenocarcinoma focal distribution, x200.

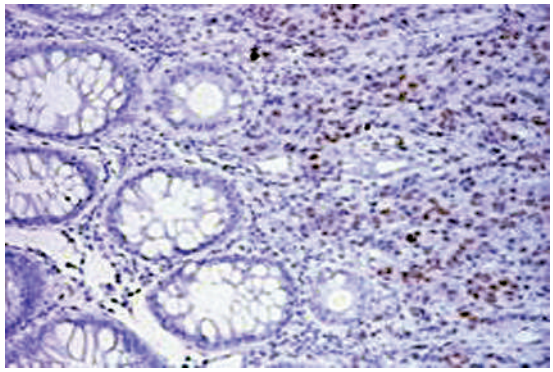

Fig.6 p53 staining in G3 colorectal adenocarcinoma diffuse distribution, x100.

p53

p53 was detected in 24 out of the 41 (58.53%) analyzed colorectal carcinomas. Only the specimens which presented 5% or more stained malignant nuclei were scored as positive, regardless of the staining intensity. A p53 positive staining with a granular or reticular pattern was localized in the nuclei of the carcinoma cells. Cytoplasmatic staining was not observed. No reactivity with anti-p53 antibody (clone DO 7) was seen in normal adjacent colonic mucosa.

The relationship between the p53 immunoexpression and the histological types of the analyzed colorectal carcinomas is shown in Table II. The percentages of positive cases increased with the histological grade in adenocarcinomas (60% in well differentiated, 61.90% in moderately differentiated and 77.77% in poorly differentiated adenocarcinomas),

Table II p53 antibody positive rate in histological type

Histological type	No.	No. of p53 positive cases (positive rate %)	
Adenocarcinoma			
grade1	5	3 (60)	**
grade 2	21	13 (61.90)	**
grade 3	9	7 (77.77)	
Mucinous adenocarcinoma	4	0	
Signet ring carcinoma	2	1(50)	

**p not significant

and was 50% in signet ring carcinomas. There was no case of mucinous adenocarcinoma to overexpress p53 oncoprotein.

The p53 overexpression was more frequently seen in adenocarcinomas (65.71% positive rate, 23 cases) than in mucus secreting cells carcinomas (16.66% positive rate, only one case of signet ring carcinoma) ($p<0.05$).

The p53 staining distribution was diffuse (i. e. more than 50% of the cells were stained) in 10 cases (41.67%) and focal (i. e. 5-50% of the cells were stained) in 14 cases (58.33%). The percentage of cases with diffuse distribution of the p53 staining was: 33.33% in grade 1 adenocarcinomas (1 case), 38.46% in grade 2 adenocarcinomas (5 cases-Fig.5) and 57.14% in grade 3 adenocarcinomas (4 cases) (Fig.6), increasing with the dedifferentiation of these tumours. The focal distribution of p53 staining was found in all the other cases of adenocarcinomas and in the signet ring carcinoma. All the cases with p53 overexpression showed high values of PCNA and Ki67 LI. The small foci of squamous metalpasia did not show any positivity to p53 oncoprotein.

Discussion

The quantification of neoplastic cell proliferation is currently the subject of a considerable number of investigations. The antibodies used to follow the proliferative activity of the cells are PCNA (proliferating cell nuclear antigen) and Ki-67 (MIB-1).

The PCNA staining technique is associated with variations in the staining intensity according to the fixing conditions, and the staining intensity may vary markedly among individual positive cells (11). In contrast, the immunohistochemical technique employing the Ki-67 antigen is simple and applicable to surgical specimens, and the reproducibility with MIB-1 antibody staining is excellent, even when paraffin-embedded tissue sections are used (12). Although the biological function of this antigen remains unclear, it is known that its expression varies according to the cell cycle; it does not appear in the resting phase G0, but does appear in all cells in the proliferative phases G1, S, G2 and M (13). The proliferating cell nuclear antigen is also involved in the DNA repair and it is known that the PCNA immunostaining may appear in cases where DNA repairs occur, but where there is no cell proliferation (4). Moreover, unlike Ki-67, PCNA may continue to be expressed in cells which have left the cell cycle, while the Ki-67 antigen is rapidly degraded (14). It is recommended to simultaneously use anti-PCNA and anti-Ki-67 antibodies in the IHC studies.

In our study, the mean PCNA LI in colorectal carcinomas was 60% (20-97%) which is in accordance with the previously published data: 54.78% ± 11.35% (15), 51.5% ± 8.2% (16). The mean Ki-67 LI was 48% in accordance with other studies (Table III).

Ki-67 LI was constantly smaller than PCNA LI because of the different immunoexpression of the two antigens (see above).

Table III Published evaluations of Ki-67 index, with prognostic relevance in colorectal carcinomas

Mean Ki67 index(%)	No. patients	Reference
38.7%	61	Porschen R, 1989 (17)
43.7%	56	Palmquist R, 1999 (18)
38.12%	52	Saleh HA., 1999 (19)
50.6%	110	Kimura T, 2000 (20)
59.42%	51	Duchrow M, 2003 (21)
32.8%	43	Ihmann T, 2004 (22)

Regarding the histological grade of adenocarcinomas, the tumor proliferative activity detected with PCNA and Ki-67 antibodies increased with the decrease of the cell differentiation. The stepwise increase of mean PCNA and Ki-67 LI with the dedifferentiation of the colorectal adenocarcinomas indicates a cell hyperproliferation in poorly differentiated adenocarcinomas, PCNA and Ki-67 being markers of the cell kinetic cycle in colorectal adenocarcinomas.

Shpitz *et al* showed that the hyperproliferation with upward expansion of the proliferative compartment is a characteristic feature in all stages of malignant progression in colorectal carcinogenesis (23).

In other malignant tumours, a high Ki-67 LI was associated with a more aggressive behavior and with a worse clinical outcome, but in colorectal tumours the relation between cell proliferation and clinical outcome is uncertain. Some authors concluded that colorectal carcinomas with low tumour cell proliferation detected with PCNA or Ki-67 had a poor prognosis (24) whereas other cases with high Ki-67 expression had a worse prognosis (20) and some studies showed no correlation between these parameters (25). In the present study we found a marked heterogeneity for the PCNA and MIB-1 expression in the same sample of colorectal adenocarcinoma, heterogeneity which was not found in normal mucosa. We consider that this finding could be an explanation for the missing relation between cell proliferation and clinical outcome in this type of tumour.

The proliferative activity detected with PCNA and Ki-67 in the foci of squamous metaplasia was lower than the proliferative activity of the malignant areas in the analyzed adenocarcinomas. Thus, the foci of squamous metaplasia, present in colorectal adenocarcinomas, seemed not to influence the increase and the extension of the tumour.

In our study, the carcinomas with mucus secreting cells had a mean PCNA LI of 50% for mucinous adenocarcinomas and 70% for signet ring carcinomas. These mean values suggest that the cell proliferation in these types of carcinomas is no higher than in moderately differentiated (PCNA LI: 53%) and, respectively, in poorly differentiated adenocarcinomas (PCNA LI: 72%). However, the mean Ki-67 LI was 32% for mucinous adenocarcinomas (similar to moderately differentiated adenocarcinomas, and 45% for signet ring carcinomas (lower than in the cases of poorly differentiated adenocarcinomas). Thus, we proved that in signet ring carcinomas, the PCNA LI and Ki-67 LI did not correlate.

Knowing that MIB-1 is a better antibody than PCNA for detecting mitotic activity, we consider that the proliferative activity in signet ring carcinomas is between the values found in moderately differentiated and poorly differentiated adenocarcinomas. Moreover, Ishida H *et al* 6) showed that the MIB-1 expression for mucinous carcinomas is significantly lower, even in comparison with MIB-1 expression for well and moderately differentiated adenocarcinomas. In our study, the proliferative activity correlated with the histological grade of the adenocarcinomas, but not with the histological type of colorectal carcinomas (poorly differentiated carcinomas, such as signet ring carcinomas, had a relatively low proliferative activity). The reason for the discrepancy between the histological type and Ki-67 LI remains unknown (6). However, these results have led us to believe that the already known poor prognosis of signet ring carcinomas, does not exclusively depend on their proliferative activity.

The p53 overexpression detected by immunohistochemistry is based on the accumulation of p53 protein in cells. But the wild p53 type can also accumulate in the nucleus in the case of cellular hypoxia or DNA alterations. Moreover, not all aberrant mutations of p53 cause p53 accumulation and this can cause false negative results (26). In colorectal carcinomas this correlation between p53 gene status and p53 immunostaining was estimated in over 70% of the cases (2).

The p53 overexpression was detected in our study in 24 cases (58.53%). In previously reported studies, p53 overexpression was observed in 60.6% (27); 67.3% (28) and 30% of the cases (29).

Regarding the cell differentiation in adenocarcinomas, we observed that the p53 positive rate increased with the dedifferentiation of these tumours. The p53 staining distribution was diffuse in 37.5% of the cases of low grade adenocarcinomas (grade 1 and 2) and in 57.14% of the cases of high grade adenocarcinomas (grade 3). Low grade adenocarcinomas presented a focal expression of p53 more frequently than high grade adenocarcinomas, which generally expressed p53 in a diffuse manner.

The high p53 positive rate in high grade adenocarcinomas together with the high rate of p53 diffuse distribution in these tumours suggest that p53 is involved in cell dedifferentiation in colorectal adenocarcinomas.

Only 16.66% of the cases of carcinomas with mucus secreting cells overexpressed p53, while the adeno-carcinomas overexpressed this protein in many more cases (65.71%). This finding is consistent with previously reported results (30). The lower frequency of p53 alteration in carcinomas with mucus secreting cells suggests that these carcinomas occur in a different way compared to adenocarcinomas.

Cell proliferation is inhibited by normal or wild-type p53 protein which acts by arresting the cell cycle at the G1-S phase in order to allow DNA repair to take place. Loss of this activity may lead to neoplastic transformation (31). In our study, the p53 overexpression was associated with the highest mean PCNA and Ki-67 LI which suggested an accumulation of a mutant p53 protein.

By correlating the results for the studied markers with the morphological aspects, we concluded that the p53 overexpression was associated with the histological grade of the colorectal adenocarcinomas and with the nonmucinos carcinomas and tended to be more frequent in the colorectal carcinomas with a high proliferative activity.

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