# Immunohistochemical Study on the Expression of P53 Protein and Proliferating Markers (KI 67 and PCNA) in Colorectal Neoplastic Lesions

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***Özet:*** *KOLOREKTAL NEOPLASTİK LEZYONLARDA P53 PROTEİN EKSPRESYONU VE PROLİFERASYON BELİRLEYİCİLERİNİN (Ki67 VE PCNA) İMMUNOHİSTOKİMYASAL ANALİZİ*

*Bu çalışmada 19 kolorektal neoplastik lezyonlu vakaya ait polipektomi materyalinde p53 protein ekspresyonu ile proliferasyon belirleyicileri Ki67 ve PCNA'in immünhistokimyasal analizi yapılda. Kontrol vakaları hafif ve orta derecede atipi ile karakterli adenomlar olup, problem vaka grubu ise borderline lezyonlar, fokal karsinom gösteren adenom vakaları ile intramukozal iyi diferansiye adenokarsinomlardı. Problem vaka grubunun tamamında p53 immünreaktivitesi pozitifti. Bu vakaların % 88.8'inde fokal immün boyanma patterni izlendi. Kontrol grubunda ise yalnızca 1 vaka (%10) fokal ve düşük yoğunlukta boyanma patterni ile pozitiflik gösterdi. p53 poitifliği ile proliferasyon belirleyicilerinin (PCNA VE Ki67) immün reaktivitesi arasında direkt bir ilişki gözlendi. Ayrıca kontrol vakalarında Ki67 ve PCNA için pozitif hücre oranları %42 ve %44 iken porblem vakalarda bu oranlar %83 ve %85 olarak saptandı. Proliferasyon belirleyicilerinin karakteristik immünhistokimyasal patternleri değerlendirildi. Kontrol vakalarında pozitif hücreler (%70) süperfisyal pattern gösterirken, problem vakaların %89'u diffüz reaksiyon patterni gösterdi. Sonuç olarak; kolorektal neoplastik lezyonlarda p53 ekspresyonu malign fenotipin belirlenmesinde oldukça spesifiktir. Ayrıca proliferasyon belirleyicileri de (PCNA ve Ki67) malign ve benign kolorektal lezyonların ayırıcı tanısında faydalı olabilmektedir.*

**Anahtar kelimeler:** p53, PCNA, Ki67, adenom, karsinom, kolorektal, immünhistokimya

***Summary:*** *We examined 19 cases of colorectal neoplastic lesions of polipectomy specimens for immunohistochemical expression of p53 protein and correlated these findings with immunodetection of proliferative markers, Ki67 and PCNA, using a control-case study. The control cases were adenomas with mild or moderate atypia and the problem cases were borderline lesions, focal carcinoma in adenoma and intramucosal well differentiated adenocarcinomas. We observed p53 positivity in all of the problem cases but focal in 88,8 % of the cases. In the control group, only a case (10%) showed positivity and was focal with low intensity. P53 positive foci demonstrated direct relationship to immunodetection of proliferating markers (Ki67 and PCNA). The ratio of the positive cells for Ki67 and PCNA was 42% and 44% in the control cases while 83% and 85% in the problem cases, respectively. In addition. characteristic immunohistochemical pattern of these markers were identified. The positive cells were mainly superficial (70 %) in the control cases, but 89 % of cases demonstrated diffuse pattern of reaction in problem cases.*

*We concluded that p53 expression is highly specific in determining of malignant phenotype in colorectal neoplastic lesions, and combinating it with proliferating markers may be useful instrument in the differential diagnosis between benign and malignant colorectal lesions.*

**Key words:**: p53, PCNA, Ki67, adenoma, carcinoma colorectal, immunohistochemistry.

During the last years, several nuclear proteins such as PCNA/cyclin, Ki67, C5F10, p53, myoncogene protein product which are expressed mainly in proliferating and transformed cells have been described (1,2). A range of genetic alterations have recently been described in colorectal cancer and its benign precursor like adenoma. Some of the changes have been seen to occur at an early stage of the neoplastic process such as hypomethylation of DNA chromosome 5q delection (3,4), but by contrary delections affecting the DDC locus on chromosome 18q and region of

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**Table I:** Group I (Control) and Group II (Problem) of Polypectomy Materials From Pathology Department of Hamamatsu University.

| Case | Sex/Age | Site/Size | Type | Atypia |
|---|---|---|---|---|
| 1 | F/48 | Col / 10x8x6 mm | TA | Mild |
| 2 | F/60 | Col / 18x14x13mm | TA | Mild |
| 3 | F/44 | Col / 15x12x13mm | TA | Mild |
| 4 | M/53 | Col / 9x8x7mm | TVA | Moderate |
| 5 | F/54 | Col / 12x12x9mm | TVA | Mild |
| 6 | F/54 | Rec / 8x8x8mm | TVA | Moderate |
| 7 | M/33 | Rec / 10x10x8mm | TA | Mild |
| 8 | F/53 | Col / 10x10x10mm | TA | Mild |
| 9 | F/53 | Col / 5x5x6mm | TA | Mild |
| 10 | F/64 | Col / 9x7x5mm | TA | Moderate |
| | | Group I | | |
| 11 | F/48 | Col / 6x4x3mm | TVA | Focal ADC |
| 12 | F/66 | Rec / 25x15x15mm | - | W.D. ADC |
| 13 | F/66 | Rec / 8x8x7mm | TA | Focal ADC |
| 14 | F/51 | Col / 26x23x16mm | TVA | Focal ADC |
| 15 | F/66 | Rec / 30x30x20mm | TVA | Focal ADC |
| 16 | F/77 | Col / 14x10x9mm | TVA | Focal ADC |
| 17 | F/74 | Col /13x10x10mm | TA | Focal ADC |
| 18 | F/45 | Rec / 8x8x7mm | - | W.D ADC |
| 19 | F/53 | Col /11x9x7mm | TA | Focal ADC |
| 20 | F/58 | Rec / 24x20x10mm | TVA | B.L.L. |
| | | Group II | | |

Col: Colon
Rec: Rectum
TA: Tubular adenoma
TVA: Tubulo-villous adenoma
ADC: Adenocarcinoma
W.D: Well differentiated
B.L.L.: Borderline lesion

the p53 gene on the 17p are found preferentially in carcinomas (4,5). P53 is a 393 aminoacid nuclear phosphoprotein which regulates normal cell growth. The level of p53 protein in normal cells and tissues are extremely low because of the short half-life of the protein which is about 15 minutes (6) and are undetectable by standard immunohistochemical staining (7). The overexpression of p53 correlates with the presence of point mis-sense mutations of the p53 gene which is located on the human chromosome 17p (3,8,9). The mutant p53 proteins have a longer half-life of several hours, this fact results in an increase in the amount of intracellular protein for it to become detectable by immunohistochemical methods (10). Overexpression of p53 protein as determined by immunohistochemistry has been demonstrated to be closely correlated with the presence of DNA mutations in the p53 gene in different types of cancer such as colorectal carcinomas (6,11,12). On the other hand, p53 expression is present in colorectal adenomas and adenomas from Familiar Adenomatosis Coli patients (4,9,13,14). One possible explanation for the observed increased number expressing detectable p53 protein is that tumors contain a greater number of cells in cycle than normal mucosa. To test this possibility, we stained paraffin embedded tissues sections for proliferating markers, PCNA and Ki67, to search the correlation with p53 patterns. The objectives of this study were; to know the immunostaining pattern for p53 and proliferatives markers, and their correlation with the biologic behaviour of colorectal neoplastic lesions. Therefore to contribute to pathological differential diagnosis in difficult cases.

## MATERIALS and METHODS

Twenty archival paraffin-embedded colorectal polypectomy tissues were obtained from Pathology Department of Hamamatsu University School of Medicine. Representative samples of polyp specimens were collected and assigned to two groups (Table I);

Group I: Ten cases corresponding to ordinary adenomas with mild and/or moderate atypia. This was the control group.

Group II: Ten cases including adenomas with focal adenocarcinoma, intramucosal well differentiated adenocarcinoma and borderline lesions, defined as adenoma with severe atypia.

The atypia of adenomas was graded in mild, moderate and severe according to Kozuka (15);

Immunohistochemical methods: Thin 3 micron slices from paraffin-embedded specimens were deparaffinized in a routine manner. They were then boiled in DDW for p53 and PCNA and in 10 m citrate buffer for Ki 67, for 15 minutes by microwave in order to improve the stainability. After that, they were kept at room temperature for 30 minutes and then in the water for cooling down.

The slides were then immersed in methanol with 0,3 % hydrogen peroxide for 15 minutes to block any endogenous peroxide activity. They were washed three times with 0,01 ml/L PBS for 5 minutes each time and treated with 10% normal rabbit serum for 10 minutes at room temperature. After washing, the slides were incubated with the primary antibody DO7 (Novocastra, UK)-p53 and MIB-1 (Immunotech, France)-Ki67 for 1 hour at room temperature in

**Table II:** P53 Expression in Groups I and II of Neoplastic Colorectal Lesions.

| P53 Expression | Group I | Group II |
|---|---|---|
| Positivity (%) | 1(10 %) | 9(100 %) |
| Intensity | | |
| Low | * | 3(33.3 %) |
| Moderate | | 1(11.1 %) |
| High | | 5(55.6 %) |
| Distribution | | |
| Diffuse | No | 1(11.2 %) |
| Focal | * | 8(88.8 %) |
| Unicellular | | 3(33.3 %) |
| Multicellular | * | 6(66.7 %) |
| Atypia | | - |
| Mild | + | - |
| Moderate | - | |
| Severe | 0 | 3 # |
| P53/Ki67 | 80 % | 60 - 95 % (av. 82.8 %) |
| P53/PCNA | 90 % | 70 -95 % (av. 85.0 %) |

# positivity in less of 0.5 % of cells

a moist chamber. The incubation time with the primary antibody PC10 (Novocastra, UK) for PCNA was 30 minutes. The optimal dilution for each of the antibodies was 1:100 for p53 and 1: 50-100 for Ki67 and 1: 150-200 for PCNA, as determined by preliminary studies. They were all diluted with 0,01 ml/L PBS containing 1% bovine serum albumin.

The Histofine method (Nichirei Co Ltd, Tokyo,Japan) was used for immunostaining. After washing in 0,01 mol/L PBS, the slides were incubeted 10 minutes at room temperature with biotinylated anti-mouse immunoglobulin. The slides were washed with 0.01 mol/L PBS for three times and then incubated at room temperature with peroxidase-conjugated streptavidin. After incubation, they were washed with PBS. A final wash was followed by immersion for about 10 minutes in a solution containing 20 mg/dl 3-3' -diaminobenzidine (DAB) and 10 ml/dl 30%-hydrogen peroxide in 0,05 % Tris-HCI buffered at pH 7.6. The slides were counterstained with Hematoxylin and dehydrated in alcohol, cleared in xylene and mounted in DPX.

One of the cases of Group II could not be evaluated for immunohistochemical staining because the carcinoma focus was not appeared in the new sections. Then 10 control cases and 9 problem cases were studied for immunodetection of p53, Ki67 and PCNA. The percentage of positive cells was evaluated in ten high powerfields.

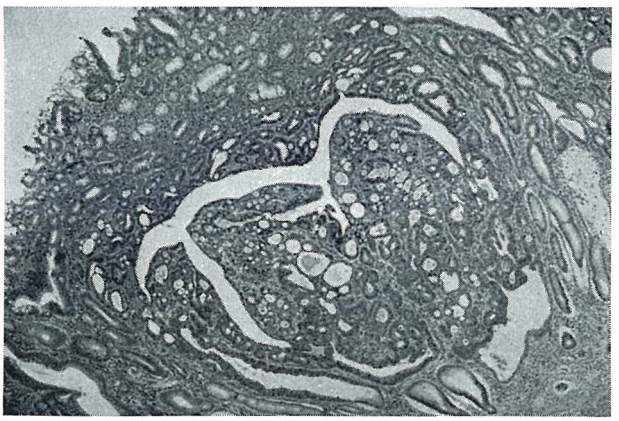

**Figure 1:** Focal intramucosal adenocarcinoma in tubular adenoma (HE x 100)

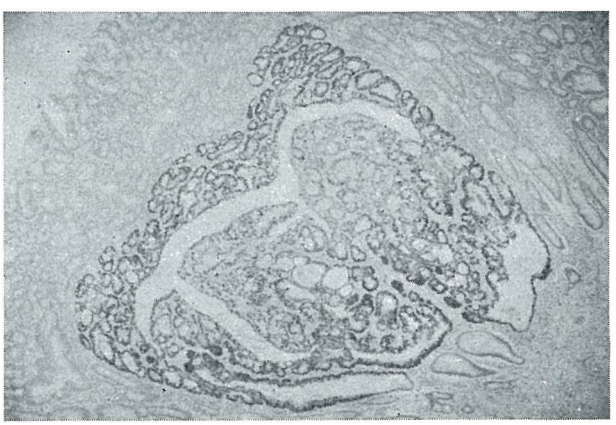

**Figure 2:** Immunohistochemical staining for p53 is strongly positivity in carcinomatous focus same with figure 1.

## RESULTS

Ninety of control cases (Group I) were negative for p53 protein. Only one case (10%) in this group presented low intensity and focal positivity for p53 (Table II). However 100% of the cases of Group II were positive for p53 and more than 50% of the cases were reactioned strongly. The distribution of the positive cells was focal in 88,8% of the cases (Fig 1,2) which was correlating with foci of carcinoma, except the borderline lesion demonstrating a focal positivity in small group of cells. One of the carcinomas presented diffuse positivity and low intensity for p53.As regard to foci of atypia, only 3 cases of severe atypia showed low positivity in less of 0.5% of the

**Table III:** Ki 67 Staining in Groups I and II of Neoplastic Colorectal Lesions.

| Ki 67 | Group I | Group II |
|---|---|---|
| Positivity (%) | 10-80% (av: 42%) | 60-95% (av: 83%) |
| Intensity | | |
| Low | 0 (0.0 %) | 0 (0.0 %) |
| Moderate | 5 (50 %) | 0 (0.0 %) |
| High | 5 (50 %) | 9 (100 %) |
| Distribution Patterns | | |
| A | 1 (10 %) | 0 (0 %) |
| B | 2 (20 %) | 8 (89 %) |
| C | 7 (70 %) | 1 (11 %) |
| Atypia | | |
| Mild | 10-30 % (av: 23 %) | 20-30 % (av: 22%) |
| Moderate | 30-80 % (av::56 %) | 30-90 % (av: 69%) |
| Severe | - | 60-95 % (av: 85%) |

cells. Only one control-case with mild atypia was positive for p53 having low intensity. In addition, the correlation of p53 positivity with staining grade for PCNA and Ki67 was searched. The only positive focus of p53 of the control cases showed positivity in 80% and 90% of the cells for Ki67 and PCNA, respectively. The intensity in this area was higher for both proliferating markers. In Group II, the p53 foci correlated with 82,8% and 85% of the positive cells for Ki67 and PCNA respectively.

The percentage of the positivity for Ki67 was 42% and 83% for Group I and II respectively (Table III). The intensity was high in 100% of problem cases while moderate or high intensity was observed in the control cases. Three patterns of Ki67 staining were detected. (Figure 3a, b. c). These were;

**Table IV:** PCNA Staining in Group I and II of Neoplastic Colorectal Lesions.

| P.C.N.A | Group I | Group II |
|---|---|---|
| Positivity (%) | 10-80% (av: 44%) | 70-95% (av: 85%) |
| Intensity | | |
| Low | 7 (70 %) | 0 (0 %) |
| Moderate | 2 (20 %) | 5 (56 %) |
| High | 1 (10 %) | 4 (44 %) |
| Distribution Patterns | | |
| A | 2 (20 %) | 0 (0 %) |
| B | 1 (10 %) | 8 (89 %) |
| C | 7 (70 %) | 1 (11 %) |
| Atypia | | |
| Mild | 10-40 % (av: 33 %) | 25-60 % (av: 39%) |
| Moderate | 40-80 % (av::62 %) | 30-90 % (av: 69%) |
| Severe | - | 75-95 % (av: 86%) |

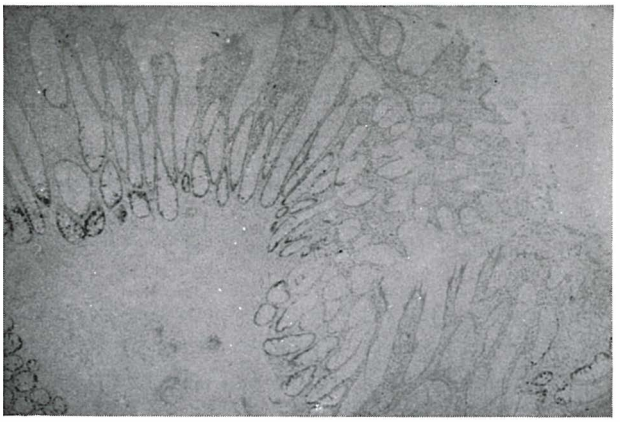

**Figure 3a:** PCNA - pattern like normal mucosa (A).

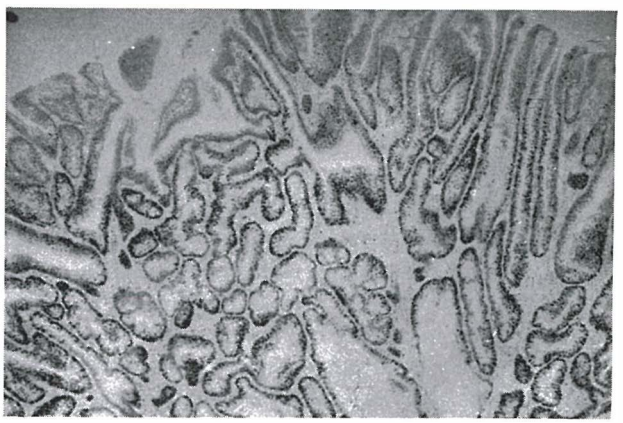

**Figure 3b:** Ki67 - difuse pattern (B).

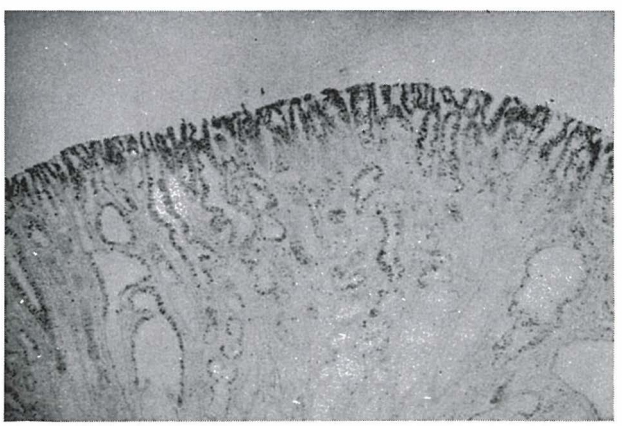

**Figure 3c:** Ki67 - inverse pattern, positivity mainly in surface.

A) Like normal mucosa, positivity was present in the deeper region,

B) Diffuse pattern,

**Table V:** Frequency of Over-Expression of p53 Protein in Colorectal Adenomas

| AUTHORS | | FREQUENCY |
|---|---|---|
| * Van Den Berg et al | (1989) | 10 % |
| * Rodrigues et al | (1990) | 0 % |
| * Pignatelli et al | (1992) | 42 % |
| * Kanklamanis et al | (1993) | 11 % / 53%# |
| * Scott et al | (1993) | 5 % |

# 11 % ordinary adenoma / 53 % severe atypia

C) Inverse pattern, positivity was present mainly in the superficial region as staining of groups of cells but positive staining of only the individual cells in the deep part.

The C pattern was the more frequent (70%) pattern observed in the control cases while B pattern (diffuse) was characteristic (89 %) in the problem cases. One of the control cases showed pattern (A) like normal mucosa, by contrary none of the problem cases showed such pattern. We observed direct correlation between the grade of atypia and percentage of positive cell especially in carcinoma and severe atypia foci. None of the neoplastic colorectal mucosa of the control cases showed usual pattern of staining (lower third of cript), but in a problem case, the normal mucosa near to carcinoma, showed B pattern for Ki67 and in the same area, scanty p53 positive cells were demonstrated (Fig 4a,b). No differences in the patterns of staining were detected relating to histologic types of adenomas.

The percentage of positivity for PCNA was frankly different in Group I (av. 44%) and Group II (av. 85%), and both groups showed clear correlation with Ki67 positivity. Seventy percentage of the control cases showed low intensity for PCNA stain, by contrary 100% of problem cases reactioned strongly (moderate to high intensity) (Table IV). Similar patterns to Ki67 were detected for PCNA staining. Two control cases (20 %) showed pattern A like normal mucosa, one case (10 %) corresponding to pattern B, but the majority of the cases showed pattern C. This distribution was equivalent to Ki67 patterns. The results for different grades of atypia also were comparable to Ki67 staining. The same problem case that showed change of Ki67 pattern in the normal mucosa near to the carcinoma have been displayed strict correlation with PCNA.

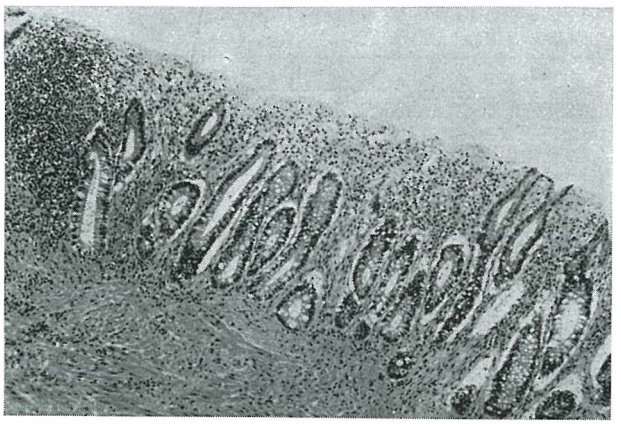

**Figure 4a:** Nonneoplastic mucosa near to the carcinoma. HE x 100

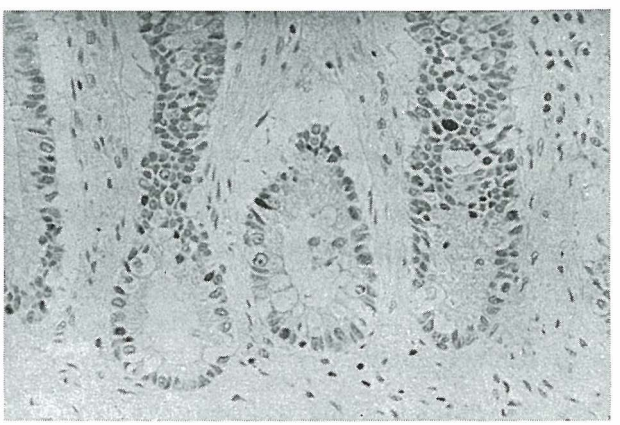

**Figure 4b:** The same area shows scanty p53 positive cells.

## DISCUSSION

All of the problem cases were positive for p53 and the p53 immunoreactivity was observed only in the nuclei of carcinomatous cells in every case. This fact confirmed the specificity of p53 protein for malignant tissue. Such finding is consistent with a strong association of p53 immunoreactivity with malignant phenotype reported in other organs (6). Only 10% of the control cases (ordinary adenomas) showed focal p53 positivity. The frequency of overexpression of p53 in colorectal adenomas, according to different reports is as follow: (Table V).

Van Den Berg et al studied 74 adenomas including 12 Familial Adenomatous Polyposis (FAP), and they found that 10 % of the sporadic and 17 % of the FAP adenomas overexpressed p53 protein (14). No clear correlation was found with

degree of atypia and no information was given pertaining to presence of malignant change. Kanklamanis et al reported that positive p53 cells were more frequent in adenomas showing a high grade of atypia (53 %) in comparison to adenomas with low atypia (11 % ) (10). Pignatelli et al observed a correlation between p53 expression and grade of atypia (9). We didn't observe p53 positive cells in mild and moderate atypia foci, however 3 cases of severe atypia showed slight staining under 0.5 % of the cells. According to Fearon et al and Vogelstein et al, these findings suggest that mutated p53 gene may play role in the late stage of colorectal tumorigenesis rather than an initial neoplastic event (4,12). P53 positive foci correlates with high reactivity of proliferating markers, Ki67 and PCNA. This relationship is not surprising, because of the p53 protein appears to inhibit cell-cycle progression into S phase. P ignatelli et al suggest that mutated p53 gene may be the direct cause of the loss of the normal proliferative controlseen in colorectal adenomas (9). On the other hand, later experiments demonstrated that the Restoration of a normal copy of p53 gene resulted in the loss of tumorigenicitiy of colon cancer cells (11). Histologically, gastric and colorectal adenomas are closely similar, however there are no reports of changes in the p53 gene in gastric adenomas (16). The results about percentage of positivity for proliferating markers were according to the Nishimura et al's results, who reported 49% and 72% of PCNA positive cells in adenoma and carcinoma respectively (17). The most frequent pattern observed in ordinaries adenomas was pattern C, however in the problem cases the principal pattern was type B (difuse). These findings correlates with the other results that demonstrates a shift of the zone of proliferation (8,18,19). Accordingly, in the neoplastic lesions the positive cells were demonstrated at relatively suface of the mucosa in adenomas, and distributed irregularly from bottom to the top in carcinomas (19). Therefore, we would like to emphasize that none of the problem cases showed like normal mucosa pattern. The direct correlation between grade of atypia and percentage of Ki67 and PCNA pasitive cells observed in this study has been previously reported (20). The p53 positivity and the change of the staining pattern for Ki67 and PCNA in the normal mucosa near to the carcinoma suggest that many colorectal carcinomas secrete growth factors (e.g. growth factor alpha) that activate gene expression. Similar findings was observed by Mehlem et al about myc expression in normal colorectal mucosa surrounding tumors (21).

In conclusion; the p53 expression is a highly specific immunostaining for carcinomatous tissue in colorectal neoplastic lesions. This study suggests that immunohistochemical demonstration of p53 protein may be a suitable method for routine detection of subpopulations of cells which, by clonal expansion could acquire a growth advantage within an adenoma during the neoplastic process. The rarity of p53 in adenomas and its prevalence in carcinomas suggest that p53 mutation is one of the late events in the adenoma-carcinoma sequence, occurring either at the adenoma-carcinoma interfase or during progression of established cancer (3,8). Staining for Ki67 and PCNA would specifically demonstrate different patterns of proliferation useful for to elucidate the biological behaviour of a colorectal neoplastic lesion. The combinated use of immunohistochemical staining for p53, Ki67 and PCNA are very useful instruments when the pathologist is up against a difficult colorectal neoplastic lesion.

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