Loss of Heterozygosity of p53 Gene and p53 Protein Expression in Human Colorectal Carcinomas

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ABSTRACT

The p53 gene is a tumor suppressor gene located on chromosome 17p. Deletions of this chromosome and point mutations of p53 have been implicated in the development of colon neoplasms. We have analyzed the loss of heterozygosity of the human p53 tumor suppressor gene in 40 cases of colorectal carcinoma using two restriction fragment length polymorphisms detected by BglII and AccI restriction enzymes. p53 gene product expression was studied immunohistochemically in 64 colorectal carcinomas, 18 adenomas, and 40 normal colorectal mucosa using an anti-human p53 monoclonal antibody (Pab 1801) and the avidin-biotin-peroxidase complex technique. Twelve of the 40 patients (30%) were polymorphic for the p53 gene. In ten of these informative patients (83%), the tumor samples showed the loss of one allele when compared with normal colorectal samples of the same patient. One of the homozygous patients showed a loss of both p53 alleles. p53 immunostaining was observed in 43 of 64 carcinomas (67%) but only in two adenomas (11%). These two positive adenomas showed areas of carcinoma in situ. The normal mucosa was always negative. No relation could be found between p53 immunostaining and the degree of differentiation, the extension of the tumor, or the Ki-67 proliferative index. Mucinous carcinomas and right-side carcinomas were less p53 immunoreactive (25% and 52%, respectively) than the usual adenocarcinomas (73%) and distal tumors (72%). These findings suggest that p53 may be a target of chromosome 17 deletions and that this gene may play a role in the malignant transformation of adenomas. BglII and AccI restriction fragment length polymorphism analysis of the p53 gene may be a useful and direct technique to detect allelic loss of this gene in tumors.

INTRODUCTION

Neoplastic transformation of cells is related to major genetic changes which include such different mechanisms as the activation of certain cellular oncoproteins and the inactivation of other types of genes called tumor suppressor genes (1–3). Malignant conversion of colorectal mucosa is a progressive process in which adenomas with different grades of dysplasia are well-known intermediate stages between normal mucosa and invasive carcinomas (4). Recently, several chromosomal alterations and the activation of different oncoproteins have been associated with the different steps of neoplastic progression in colorectal mucosa (5–12).

p53 is a nuclear protein initially described as a protein associated with the simian virus 40 large tumor antigen (13). This protein may play a certain role in the regulatory control of normal cell proliferation (14–17). The p53 gene has also been implicated in neoplastic transformation of experimental and human cells (16, 18, 19). Although initially p53 was considered an oncogene, recent studies suggest that the p53 gene might act as a tumor suppressor gene (20, 21).

The human p53 gene has been located on chromosome 17p (22). Allelic deletions of this chromosome occur in more than 75% of colon neoplasms but rarely in adenomas (9). The region of the chromosome commonly lost seems to contain the p53 gene (23). However, several studies of these neoplasms have not demonstrated gross alterations of the p53 gene (23–26) nor of the p53 mRNA transcript (23, 27). Point mutations of this gene have been observed in the remaining allele of colon carcinomas with chromosome 17 deletions (23, 28).

Mutant p53 is metabolically more stable than the wild type (19) and thus may be more expressed in neoplastic than in normal tissues (29). Recent studies have suggested that the p53 detected immunohistochemically in tumors and cell lines actually corresponds to a mutated form of the protein and not to the wild type (29, 30). Thus, this technique may be useful to correlate the presence of p53 mutations with the clinicopathological characteristics of the tumors.

The existence of gene loss can be analyzed by the use of RFLPs that show the deletion of discrete chromosomal regions from tumor DNA compared with the DNA of normal tissues from the same patient. The BglII restriction enzyme identifies a two-allele polymorphism within the human p53 locus with polymorphic bands at 12 and 9 kilobases as well as a constant band at 3.2 kilobases (31, 32). The AccI restriction enzyme identifies a two-allele polymorphism within exon 4 of the human p53 gene. The A1 allele shows a unique band of 259 base pairs, and the A2 allele presents a two-band pattern of 160 and 90 base pairs (33–35).

In this study we have analyzed the loss of heterozygosity of the human p53 gene in colorectal carcinomas by the use of these recently described RFLPs and have immunohistochemically examined p53 expression in normal and neoplastic colorectal mucosa. The findings were correlated with several clinical and pathological parameters of the tumors.

MATERIALS AND METHODS

Tissues. Sixty-four surgically removed colorectal carcinomas, 18 adenomas, and 40 normal mucosa were examined. The histological classification of the tumors was established according to previous criteria (36). Thus, 4 were well-differentiated adenocarcinomas (6%), 43 were moderately differentiated adenocarcinomas (67%), 9 were poorly differentiated adenocarcinomas (14%), and 8 were mucinous carcinomas (13%). The distribution of these tumors according to Dukes' system was Dukes' Stage A, 7 cases (11%); Dukes' Stage B, 21 cases (33%); and Dukes' Stage C, 36 cases (56%). Sixteen cases (25%) were located in the proximal colon, and 48 cases (75%) were in the distal colon. "Proximal colon" specimens included those from the cecum and ascending and transverse colon, whereas descending, sigmoid, and rectal specimens were included as "distal colon." Adenomas were

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: RFLP, restriction fragment length polymorphism; cDNA, complementary DNA; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; ABC, avidin-biotin-peroxidase complex.
obtained from both surgically removed specimens and fiberoptic biopsies. Dysplasia in adenomas was graded as mild, moderate, and severe/ carcinoma in situ according to previous criteria (36). Normal mucosa samples were selected from macroscopically normal areas of surgical specimens. One sample was taken from more than 10 cm away from tumors and another one from normal mucosa adjacent to carcinomas (transitional mucosa).

Samples for genetic and immunohistochemical studies were snap frozen in isopentane precooled in liquid nitrogen and stored at -80°C until studied. The remaining specimen was fixed in formalin overnight and routinely processed.

Probes. pLA65 (pArgSP53) is a recombinant cDNA clone that contains the entire coding region of the human p53 gene (34). This probe was kindly provided by Dr. Crawford at the Imperial Cancer Research Foundation, Cambridge, United Kingdom.

DNA Extraction and Southern Blotting. Forty carcinomas and their normal mucosa far from the tumor were submitted for DNA extraction and Southern blot studies. Cryostat sections of these cases were previously examined in order to determine the proportion of normal and neoplastic tissue present in each sample. Tumor samples were selected only if more than 80% of the section was carcinomatous. DNA of each sample was extracted by conventional methods, and 10 μg was digested with BglII, separated on 0.8% agarose gels, and transferred to Hybond-N membranes (Amersham) according to the method of Southern (37). The membranes were prehybridized with 50% formamide, 5× SSC, 5× Denhardt’s, and 500 μg/ml of denatured salmon sperm DNA at 42°C for 16 h and hybridized with 50% formamide, 5× SSC, 1× Denhardt’s, 100 μg/ml of salmon sperm DNA, 10% dextran sulfate, and 100 cpm/ml of 32P-labeled human p53 probe for 24 h. After hybridization, membranes were washed with 2× SSC and 0.1% SDS at room temperature for 30 min followed by 2× SSC and 0.1% SDS at 60°C for 30 min and 0.1× SSC and 0.1% SDS at 60°C for 1 h. The filters were then autoradiographed using intensifying screens at -70°C for 24 to 72 h (38).

Polymerase Chain Reaction Amplification. The fourth exon of the human p53 gene was amplified PCR (39), using the sense oligonucleotide 5'-AATGGATGATTTGATGCTGTCCC-3' and the antisense oligonucleotide 5'-CGTGCAAGTCACAGACTTGGC-3'. PCRs were carried out in a total volume of 50 μl containing 500 ng of genomic DNA, 50 pmol of each primer, 2 mM MgCl₂, 200 μM deoxyribonucleoside triphosphates, 50 mM KCl, 20 mM Tris-Cl (pH 8.3), and 0.1% gelatin. The amplification was performed for 35 cycles with an annealing temperature of 62°C. The amplified DNA was digested overnight with a 10-fold excess of AccII. DNA fragments were resolved by electrophoresis through a 2% agarose gel or a 7% polyacrylamide gel.

Immunohistochemistry. Frozen tissues were cut at 6 μm on a cryostat. Sections were air dried and fixed in cool (4°C) acetone for 10 min.

Immunohistochemical staining was carried out using the ABC technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) (40). Slides were incubated with the primary antibody for 1 h at room temperature followed by biotinylated goat anti-mouse immunoglobulin G and the avidin-biotin-peroxidase complex for 30 and 45 min, respectively. Slides were washed 3 times with phosphate-buffered saline after each incubation. Peroxidase activity was developed by a solution of 5 mg of 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) dissolved in 10 ml of tris buffer (0.05 M, pH 7.6) and 0.03% H₂O₂. The 3,3’-diaminobenzidine tetrahydrochloride solution was filtered, and the sections developed under microscopic control. Harris’ hematoxylin was used to counterstain the slides. Control slides were used to replace the primary antibody with a nonrelated monoclonal antibody.

The primary monoclonal anti-human p53 antibody used in this study was Pab 1801, kindly supplied by Dr. Crawford (Imperial Cancer Research Foundation, Cambridge, United Kingdom). The pattern of immunoreactivity was considered as “negative” when no positive cells were found, “occasional” when only some isolated positive cells were identified, “focal” when wide clusters of positive cells were seen in some areas of the tumor but other regions were negative, and “diffuse” when sheets of positive cells were found throughout most areas of the tumor. In addition, the percentage of positive cells was determined in each positive case by counting a minimum of 1000 cells selected by a random method previously described (41).

In addition, 35 carcinomas were also analyzed with the Ki-67 monoclonal antibody (Dakopatts, Copenhagen) (42) to study the relation between the p53 immunoreactivity of the tumors and their proliferative index. The Ki-67 proliferative index (percentage of Ki-67-stained cells) of each tumor was determined by counting a minimum of 1000 cells as described for p53 (41).

RESULTS

Twelve of 40 cases studied (30%) were polymorphic for the p53 gene using BglII and AccII restriction enzymes. Among these 12 informative patients, one was polymorphic only for the BglII RFLP, 7 were polymorphic only for the AccII RFLP, and 4 cases were polymorphic for both BglII and AccII RFLPs. In 10 of these 12 informative patients (83%), the tumor samples showed a loss of heterozygosity of the p53 gene when compared with normal colorectal mucosa samples from the same patient (Figs. 1 and 2).

In 7 cases (Cases 2931, 3843, 5074, 6030, 7330, 7753, and 8867), the allelic loss was complete, suggesting that it affected all the cells within the sample. In 3 cases (Cases 3695, 7291, etc.)
Fig. 2. Loss of heterozygosity at the p53 gene locus in colorectal carcinoma patients. Accll RFLP analysis. Genomic DNA samples of colorectal tumor (T) and normal mucosa (M) from the same patient were extracted. p53 exon 4 was amplified by PCR, digested with Accll, and resolved by electrophoresis through a 2% agarose gel. The heterozygous pattern [259, 160, and 99 base pairs (bp)] present in Patients 5074, 7330, and 6030 disappeared in the tumor samples, suggesting a p53 allele deletion in these patients. No loss of p53 heterozygosity was detected in Patient 5133.

Table 1 Correlation between the clinical and pathological characteristics of the informative patients with colorectal carcinomas and their immunohistochemical and genetic findings

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>Localization</th>
<th>Size (cm)</th>
<th>Histological type</th>
<th>Dukes' stage</th>
<th>LN</th>
<th>p53 (%)</th>
<th>Normal mucosa</th>
<th>Tumor</th>
<th>Normal mucosa</th>
<th>Tumor</th>
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<tr>
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<td>P</td>
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<td>PD</td>
<td>B</td>
<td>-7</td>
<td>12</td>
<td>12/9</td>
<td>12</td>
<td>A2</td>
<td>A2</td>
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<tr>
<td>2931</td>
<td>80/F</td>
<td>D</td>
<td>7.0</td>
<td>MD</td>
<td>C</td>
<td>2/16</td>
<td>72</td>
<td>12/9</td>
<td>12</td>
<td>A1/A2</td>
<td>A2</td>
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<tr>
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<td>D</td>
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<td>MD</td>
<td>C</td>
<td>7/12</td>
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<td>C</td>
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<td>C</td>
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<td>70</td>
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<td>12/9</td>
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<td>MD</td>
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<td>12/9</td>
<td>12</td>
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<td>A1/A2</td>
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<td>MD</td>
<td>A</td>
<td>-7</td>
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<td>12/9</td>
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<td>MD</td>
<td>C</td>
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<td>8867</td>
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<td>PD</td>
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<td>63</td>
<td>12/9</td>
<td>9</td>
<td>A1</td>
<td>A1</td>
</tr>
</tbody>
</table>

* P, proximal colon; D, distal colon.
* PD, poorly differentiated adenocarcinoma; MD, moderately differentiated carcinoma.
* LN, number of tumor-involved lymph nodes/total of lymph nodes examined.
* Percentage of positive cells in the tumors.
* BglI and Accll identify two allelic polymorphisms, 12/9 and A1/A2, respectively.
* Cases where the allelic loss in the tumor sample was not complete.

and 8118), the allelic loss was not complete since a faint residual signal was still present, indicating the existence of contaminating normal cells or subpopulations within the same tumor of cells at different stages of progression. Only 2 of the 12 informative patients (17%) (Cases 3056 and 5133) showed no loss of heterozygosity of the p53 gene. In some polymorphic cases for BglI, the intensity of the constant band at 3.2 kilobases did not change in spite of the loss of one of the two polymorphic bands. This may suggest the presence of intragenic deletions involving the 5' region of the locus.

Case 1089 was homozygous for the 12-kilobase BglI polymorphic band and showed a very faint signal in the tumor sample when compared with its normal mucosa counterpart. This was confirmed with a very weak amplification by PCR for the Accll RFLP. This case, then, may represent a p53 homozygous loss. The hybridization pattern of the tumor sample suggests that one of the two 12-kilobase alleles has been lost because of deletion of the entire gene. The remaining allele may have undergone an intragenic deletion affecting the 5' region of this locus, since the almost nonexistent 12-kilobase band is accompanied by a nonaffected 3.2-kilobase band that shows half the signal detected in the normal mucosa sample. This tumor was immunohistochemically negative for p53.

In Cases 2931, 5074, and 8867, the loss of one of the polymorphic bands in the tumor sample was associated with an increase in the signal of the remaining allele, while the constant band was unaffected. This pattern may represent the loss of one allele and the duplication of the remaining allele because of mitotic nondisjunction during cell proliferation.

No rearrangements or gene amplifications were detected. The relation between the loss of heterozygosity, the immunohistochemical findings, and the characteristics of the tumors is summarized in Table 1.

An intense p53 immunostaining was observed in 43 of 64 colorectal carcinomas (67%). The immunoreaction was always localized in the nucleus of neoplastic cells (Fig. 3). No reactivity was observed in normal mucosa, transitional mucosa, or stromal cells. Positive neoplasms showed a diffuse immunostaining throughout the tumor with only small clusters of negative cells. Only three cases revealed a focal positivity for p53 with wide nonreactive areas (Fig. 4). The remaining 21 cases (33%) were completely negative. No cases with only occasional positive cells were found.

Only 2 of 18 adenomas studied showed a focal immunoreactivity for p53 protein. These two cases were adenomas with areas of severe dysplasia/carcinoma in situ. The nuclear immunostaining pattern in these cases was similar to that observed in carcinomas. The 9 adenomas with mild dysplasia and 7 with moderate dysplasia were completely negative.

The relation between p53 immunodetection and the histolog-
Fig. 3. p53 immunostaining of a colorectal carcinoma and normal adjacent mucosa. Immunoreactivity is localized in the nucleus of the tumoral cells, while the normal mucosa is negative. The stained cells observed in the stroma are polymorphonuclear cells in which endogenous peroxide activity was not blocked. The control section showed a similar staining in these cells, but the tumoral cells were negative. Hematoxylin-ABC, ×150.

Fig. 4. p53 immunostaining of a colorectal carcinoma. Immunoreactivity is observed focally in the tumor. In the positive area, almost all the cells are positive, while in the negative area, no stained cells are seen. Hematoxylin-ABC, ×100.

The clinical classification of the carcinomas is shown in Table 2. No differences were observed between p53 reactivity and the degree of differentiation of tumors. Mucinous carcinomas, however, were less frequently positive (25%) than the usual adenocarcinomas (73%) ($P < 0.05$).

The relation between p53 immunoreactivity and Dukes' stage is shown in Table 3. The number of positive cases tended to decrease in more advanced tumors, but the differences were not statistically significant. There appeared to be less p53 positive tumors in proximal colon (56%) than in distal mucosa (71%). The difference, however, was not statistically significant.

The number of positive cells in the p53 immunoreactive cases ranged from 27% to 81% (65 ± 14). According to the percentage of immunoreactive cells, the positive neoplasms were classified as low (<40%), intermediate (40 to 69%), or high (≥70%) expression tumors (Fig. 5). Most of the tumors (91%) showed an intermediate or high expression of the protein. Only 4 of the 43 positive cases (9%) had less than 40% positive cells, and 2 of these were Dukes' Stage A tumors. The proportion of p53 positive cells tended to increase during the progression of the neoplasm. Fifteen of 22 (68%) of Dukes' Stage C tumors had more than 70% positive cells, while only 6 Dukes' Stage B (40%) and 2 Dukes' Stage A (33%) tumors showed a similar level of expression.

Twenty-eight of 35 tumors studied with Ki-67 antibody expressed p53 immunoreactivity. The staining was seen in the nucleus with a granular pattern (Fig. 6). The distribution of positive cells was irregular with some areas showing a high
domains (51-55). Point mutations in the p53 gene have been
found in 11% of adenomas. Van den Berg et al. (50), using a different monoclonal antibody, found p53 expression in 55% of colorectal carcinomas but only in 8% of adenomas (11%). These two cases had morphological changes of severe dysplasia/carcinoma in situ. Only 3 cases showed focal immunostaining in which the positive number of immunostained cells while in other fields the number was very scarce. The mean percentage of Ki-67-labeled cells in the 28 p53 positive tumors (29.8%) was similar to that observed in the 7 p53 negative ones (29.2%).

DISCUSSION

The deletion of specific chromosomal regions has been described to be an important step in the development of various human malignancies (10, 12, 43-46). This phenomenon often appears to involve the loss of normal alleles that masked the effect of mutations affecting the other allele. This mechanism has been suggested for tumor suppressor genes such as p53 (23, 28, 29).

In this study we have analyzed the loss of heterozygosity of the human p53 suppressor gene in 40 colorectal carcinomas using two recently described RFLPs detected by BglII and AcclI restriction enzymes, that directly involve the p53 locus (31-35). At least 12 cases (30%) were polymorphic for the p53 gene, and in 10 (83%) of these patients, the tumor showed the loss of one allele. Another patient (Case 1089), homozygous for the 12-kilobase BglII polymorphism, showed a nearly total loss of the remaining allele (Table 1).

The high frequency of loss of heterozygosity of the p53 gene in our series is very similar to the number of chromosome 17p deletions (75%) observed in colonic carcinomas (9, 49) and suggests that the loss of this gene plays an important role in the pathogenesis of these neoplasms. Allelic loss of chromosome 17p has been associated with a poor prognosis in colorectal tumors (49). The RFLPs used in our study may be useful to determine whether the poor prognosis of such deletions is related to the specific loss of the p53 gene.

We also observed a clear relation between the expression of p53 and the malignant transformation of colonic mucosa. p53 immunoreactivity was detected in 43 of 64 (67%) carcinomas. Normal mucosa, however, was always negative, and only two adenomas (11%) showed a focal reactivity. These two cases had morphological changes of severe dysplasia/carcinoma in situ. Van den Berg et al. (50), using a different monoclonal antibody, found p53 expression in 55% of colorectal carcinomas but only in 8% of adenomas.

Transforming action of p53 has been associated in vitro with the presence of point mutations in highly conserved protein domains (51-55). Point mutations in the p53 gene have been detected in several human tumors including colorectal carcinomas (23, 28, 29, 56). Previous studies have shown that the mutated form of p53 is much more stable than the wild type, due in part to an increase in the half-life of the protein itself and the formation of stable complexes between mutated and wild p53 as well as with other cellular proteins (19, 52, 53). Although we did not establish in our study whether the p53 that was immunohistochemically detected corresponds to a wild or mutated type, the overexpression of p53 in carcinomas may be due to the presence of a more stable mutated protein. Iggo et al. (29) and Rodrigues et al. (30) have recently shown that when p53 is detected immunohistochemically in human lung tumors and in colorectal cancer cell lines, respectively, it is actually a mutated form. All these findings suggest that, in the neoplastic process of colorectal mucosa, the mutation of the p53 gene may be closely associated with the malignant transformation of adenomas. In carcinomas, the frequency of p53 gene alterations seems to be very high. In the subset of 12 informative patients in our series, only one patient showed neither immunohistochemical evidence of p53 nor loss of heterozygosity. In 8 of the 12 informative patients (67%), loss of heterozygosity was associated with an increase in the expression of the protein, suggesting the presence of a mutation in the remaining allele (Table 1).

The human p53 gene has been localized to chromosome 17p (22). The p53 genetic and immunohistochemical findings in the present series have certain similarities with the pattern of allelic deletions of chromosome 17 observed in colorectal carcinomas in previous studies (9, 49, 57). Loss of this chromosome has been detected in 77% of colonic carcinomas but only in 6% of adenomas (9). Chromosome 17 deletions were found less frequently in mucinous and right-side colonic neoplasms than in the more common adenocarcinomas and distal tumors (49, 57). The frequency of p53 allelic loss in our series (83%) was similar to that reported for chromosome 17. Similarly, p53 immunostaining was observed predominantly in carcinomas rather than in adenomas, in nonmucinous adenocarcinomas, and in left-side neoplasms. These relations suggest that both phenomena, chromosome 17p deletions and p53 expression, may be related.

An interesting finding was the homogeneous diffuse pattern of reactivity observed throughout the neoplasm in most tumors. Only 3 cases showed focal immunostaining in which the positive areas were very well delineated from the negative ones. Similarly, only 4 cases (9%) showed a low percentage of p53 im-

Table 2 p53 immunoreactivity in 64 colorectal carcinomas

<table>
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<th>Histological type</th>
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<th>%</th>
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<td>11</td>
</tr>
<tr>
<td>Carcinomas</td>
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<td>43</td>
<td>67</td>
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<tr>
<td>Well differentiated</td>
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<tr>
<td>Poorly differentiated</td>
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Table 3 Comparison of p53 immunoreactivity in 64 colorectal carcinomas and their Dukes' stage

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<td>86</td>
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<tr>
<td>B</td>
<td>21</td>
<td>15</td>
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</tr>
<tr>
<td>C</td>
<td>36</td>
<td>22</td>
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Fig. 5. Correlation between the number of p53 immunoreactive cells in the 43 positive cases and Dukes' classification. The number of positive cases in Dukes' Stages A, B, and C is plotted against the percentage of positive cells.
munoreactive cells. The number of positive cells tended to increase with the progression of the neoplasms. This suggests that p53 alteration in carcinoma cells might provide the cells with a certain growth advantage allowing their clonal expansion.

p53 has been implicated in the control of cell division and proliferation (14–17). Proliferative activity of normal and neoplastic tissues may now be immunohistochemically assessed by means of the Ki-67 monoclonal antibody (42). This antibody recognizes a nuclear protein expressed in all phases of the cell cycle except G0. In our study we observed that the expression of p53 in colonic carcinomas does not appear to be related to the proliferative activity of tumors, since the number of cells expressing p53 positive and negative neoplasms was similar.

In summary, this study shows a high frequency of alterations of the p53 gene in human colorectal carcinomas. Most of the tumors have an allelic loss of the gene and, possibly, a mutation of the remaining allele leading to a high expression of the protein. The immunohistochemical findings suggest that the mutation of p53 may be involved in the malignant transformation of adenomas. The use of BgII and AcII RFLPs is a direct technique to study the allelic loss of the p53 gene in human tumors.

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p53 IN COLORECTAL CARCINOMAS


Loss of Heterozygosity of p53 Gene and p53 Protein Expression in Human Colorectal Carcinomas

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