A Frequent Alteration of p53 Gene in Carcinoma in Adenoma of Colon

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ABSTRACT

In general, colorectal carcinoma is thought to originate mainly from adenoma, and this pathway is called the adenoma-carcinoma sequence. Carcinoma in adenoma is an appropriate model for analysis of this mechanism, because adenoma and carcinoma tissues coexist in the same polyph and the carcinoma is thought to have originated from the surrounding adenoma. Expression of the p53 protein was analyzed in 36 cases of carcinoma in adenoma in the colon by immunohistochemistry using an anti-human p53 monoclonal antibody (PAb1801). Alterations of the p53 gene were analyzed by the polymerase chain reaction for microanalysis of normal mucosa, adenoma, and carcinoma from histological slides. Mutations were assessed by polymerase chain reaction-single strand conformation polymorphism analysis and identified by DNA sequencing in some cases. Loss of heterozygosity was studied by polymerase chain reaction-restriction fragment length polymorphism analysis. Positive staining for p53 was detected in three (8%) of 37 adenomas and 20 (53%) of 38 focal carcinomas. One (7%) of 15 adenomas with mild dysplasia, three (14%) of 22 adenomas with moderate dysplasia, and 16 (42%) of 38 focal carcinomas had a mutation in exon 5 through exon 8 of the p53 gene. As for allelic loss in the p53 gene locus, only one adenoma with moderate dysplasia had loss of heterozygosity, whereas six (40%) of 15 focal carcinomas had loss of heterozygosity. Of those tumors (3 of 37 adenomas and 20 of 38 focal carcinomas) that reacted with PAb1801, 78% (18 of 23) showed genetic alterations. Among 52 tumors which showed negative staining, five tumors had a p53 mutation and four of them were nonsense mutations. Putting all of these results together, 71% (24 of 34) of the cases underwent p53 gene alterations. Among 52 tumors which showed negative staining, five tumors had a p53 mutation and four of them were nonsense mutations. Putting all of these results together, 71% (24 of 34) of the cases underwent p53 gene alterations. Among 52 tumors which showed negative staining, five tumors had a p53 mutation and four of them were nonsense mutations.

INTRODUCTION

Mutations of the p53 gene are the most frequently observed genetic change in human cancers (1, 2). Various point mutations within the coding region of this gene can convert the normal p53 gene to a dominant oncogene by yielding the mutated form of p53 (3-5). Previous reports have suggested that mutation of the p53 gene is a common occurrence associated with allelic loss on chromosome 17p in carcinogenesis in various tissues (1, 6, 7).

Abnormal expression of the p53 protein has been studied by many investigators using immunohistochemical analysis. Mutant forms of the p53 protein can bind to the wild-type form, thereby stabilizing its rapid degradation and extending its half-life (8, 9). Exceptionally, accumulation of the wild-type p53 protein was reported in some cases of human astrocytoma (10). In general, however, immunohistochemically detectable p53 protein has been widely accepted as reflecting an underlying mutation or LOH (11-19).

Recently, many reports have suggested that the accumulation of multiple gene alterations is associated with multiple steps of carcinogenesis in colorectal carcinoma (20-29). This concept is based on the scenario called “adenoma-carcinoma sequence.” In recent years, however, many epidemiological and histopathological studies have appeared to indicate the existence of another mechanism called “de novo” colon carcinogenesis (30-32). Today, there is much controversy regarding these two pathways. That is, which pathway is actually dominant in colon carcinogenesis, the adenoma-carcinoma sequence or the de novo mechanism? Most of the previous reports on p53 gene alteration in colorectal carcinomas did not take into account the differences in these proposed pathways. Alteration of the p53 gene is suspected to be a late event in colon carcinogenesis (7, 19). However, it is still not clear whether p53 gene alteration occurs in malignant conversion or progression of carcinoma in the late stage of colon carcinogenesis.

In an attempt to elucidate alterations of the p53 gene associated with the pathway based on the adenoma-carcinoma sequence, we analyzed 36 cases of carcinoma in adenoma of the colon. This analysis, we applied the MW-fixation technique, which has been reported to achieve good fixation for preserving the DNA and the antigenicity of labile antigens (33-35). We also used a modified microanalysis technique (36) in order to analyze relatively pure cell subpopulations.

MATERIALS AND METHODS

Tissue Preparation. We examined 36 cases of carcinoma in adenoma of the colon. The tissues were obtained from 32 patients who underwent endoscopic polypectomy and two patients who underwent surgery. Two patients had two separate polyps in the colon. We trimmed all tissues before fixation. Specimens thus prepared were fixed immediately by MW irradiation according to the method as described previously (35). Briefly, each specimen was soaked in a dilute aldehyde solution [2% (v/v) formaldehyde, 0.05% (mol/liter) cacodylate buffer, and 0.0025% (v/v) calcium chloride, pH 7.35] and irradiated with 2450 MHz MW energy at 500 W for about 20 s. The specimens were then dehydrated in graded ethanol at 4°C, embedded in paraffin, and stored at 4°C to prevent degradation. Two series of 4-μm-thick sections were prepared. One section was stained with hematoxylin and eosin. Tumors were classified into three types, i.e., adenoma with mild dysplasia, adenoma with moderate dysplasia, and focal carcinoma (severe dysplasia) on the basis of the histopathological diagnosis. The other section was analyzed by immunohistochemical staining for the p53 protein. For DNA extraction, four series of 8-μm-thick eosin-counterstained sections were prepared from adjacent sections. In order to minimize the DNA damage due to staining, hematoxylin was not used.

Immunohistochemistry. A modification of the immunoglobulin enzyme bridge technique (avidin-biotin-peroxidase method) was used as described previously (35). Primary anti-p53 antibody PAbl801 (Novocastra Laboratories, Ltd, Newcastle, United Kingdom), which recognizes an epitope between amino acids 32 and 79 on human p53 protein, was applied to the sections at a dilution of 1:50. As a negative control, normal mouse serum was used in each staining.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: LOH, loss of heterozygosity; MW, microwave; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.
DNA Extraction. By comparing hematoxylin and eosin-stained sections and immunohistochemically stained sections, tissues of normal mucosa (N), adenoma (A), and carcinoma (T) were picked out from adjacent sections with a needle under a microscope (Fig. 1, a and b). In order to avoid, as thoroughly as possible, contamination with nonneoplastic cells such as inflammatory cells or stromal cells, only tumors containing ≥90% tumor cells were used. Moreover, samples with tumor cells of different grades of atypia were excluded to avoid ambiguous results in the DNA analysis. When tumors were positive for immunostaining, only tumors containing ≥50% positive-staining cells (staining frequency ≥50%) were used. In the case where heterogeneous staining was observed in adenoma or carcinoma, both of the tumors were used, as shown in Table 1 (A1, A2 or T1, T2, respectively). The size of tissue samples thus prepared varied from 0.35 to 34 mm². Then the tissue was transferred to a 500-μl microtube for PCR. After deparaffinization in xylene, the DNA was prepared by digestion with proteinase K, extraction with phenol/chloroform, and precipitation with ethanol, as described previously (37). Consequently, DNA samples of 36 normal mucosae, 37 adenomas, and 38 carcinomas were obtained from 36 cases.

Detection of LOH in p53 Gene by PCR-Restriction Fragment Length Polymorphism Analysis. BamHI polymorphism in the 3' flanking region of the p53 gene (38) was analyzed using nested PCR. First, PCR amplification was performed to generate a 188-base pair fragment using outer primers. With about 1/10 (v/v) of the first PCR products, a second PCR amplification was performed to generate a 90-base pair fragment using inner primers. The sequences of both primers were provided by Dr. Y. Nakamura. The conditions of each PCR were the same. The PCR incubation mixture was first heat denatured for 10 min, and then 2.5 units of Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT) were added. One cycle of PCR consisted of 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C; a total of 35 cycles of PCR were performed. Ten μl of PCR-amplified fragments were digested with 10 units of BamHI for 12 h at 37°C and then fractionated on a 10% polyacrylamide gel. The amplified DNA fragments were visualized by staining with ethidium bromide. BamHI digestion of the amplified fragments identified two alleles, allele 1 with 90 base pairs and allele 2 with 59 base pairs plus 31 base pairs. The outer primers used were 5'-AATTTTGCTGTTGGGGACTCATGGG-3' (upstream) and 5'-TA-GACCTAGCCCCATTCTCCAACCA-3' (downstream); the inner primers used were 5'-TCGAAAGGAAATGGAGAGGACTCAG-3' (upstream) and 5'-TGTTACACCTATAACCGAGGGCCTCCTC-3' (downstream).

Detection of Mutations in p53 Gene by PCR-SSCP Analysis. First, exons 5–8 of the p53 gene were amplified by PCR using the primers as described previously (39). Then 1.5 μl of 1/10 (v/v) of PCR-amplified products were added to 1.5 μl of a mixture of deoxynucleotide triphosphate (60 μM), 32P-end-labeled primers (0.1 μM each), MgCl₂ (1.5 mM), Tris-HCl (pH 8.3, 10 mM), KCI (50 mM), and 0.1 units of Taq polymerase. PCR amplification was then performed for 20 cycles. The annealing temperatures were 60, 64, 62, and 62°C for exons 5, 6, 7 and 8, respectively. The SSCP analysis was done as described previously (40), with the following modifications. Briefly, the PCR product (3 μl) was diluted 10-fold with stop solution (20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) and heat-denatured at 98°C for 5 min. One μl of this mixture was loaded onto an 8.3% nondenaturing acrylamide gel (acrylamide:methylene-bis-acrylamide, 80:1) and run at a constant voltage of 400 V at room temperature for 12–16 h. The gel was then vacuum-dried and exposed to Kodak X-OMAT film at room temperature for 1–2 days.

Cloning and Sequencing of PCR Products. Sequence determination of exons 6 and 8 of the p53 gene was performed. To facilitate the cloning of PCR products, the primers involving extraneous EcoRI or HindIII recognition sites, which are underlined below, were used as described previously (41). The primers for exon 6 were 5'-GTAAGGAATTCCCTTCTTCGAGTACCTC-3' (upstream) and 5'-CTCAAGCTAGTTGGAAACCGAGCTCAG-3' (downstream). The primers for exon 8 were 5'-GTAAGGAATTCCCTTCTTCGAGTAGTGTTAA-3' (upstream) and 5'-CTCAAGCTAGTTGGAGTCAGCCTGACGG-3' (downstream). The PCR products were double-digested with EcoRI and HindIII and fractionated by electrophoresis through a 2% agarose gel. The DNA fragments were eluted from excised gel slices with the aid of DNAse I (Boehringer-Mannheim, Mannheim, Germany).

**Fig. 1. p53 immunostaining, mutation detected by PCR-SSCP, and sequencing in Case 12. a. Immunohistochemical staining of p53 in carcinoma in adenoma. N, normal mucosa; A, adenoma; T, focal carcinoma. b. The shifted band which shows the probable mutation in exon 8 of the p53 gene. N, normal mucosa; A, adenoma; T, focal carcinoma. c. Sequence analysis of variant PCR product in exon 8. Arrow, the shifted band which shows the probable mutation in exon 8 of the p53 gene. d. Sequence analysis of variant PCR product in the focal carcinoma.**

*Y. Nakamura, personal communication.*
Table 1 List of individual tumors according to immunoreactivity with PAb1801

<table>
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<tr>
<th>Case</th>
<th>Dysplasia</th>
<th>Immuno-staining</th>
<th>Mutation</th>
<th>LOH</th>
<th>Immunostaining</th>
<th>Mutation</th>
<th>LOH</th>
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<tr>
<td>7</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>Ex5</td>
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<tr>
<td>44</td>
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<td>–</td>
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<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>48</td>
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<td>50</td>
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<td>+</td>
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<td>–</td>
<td>–</td>
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</table>

* CIA: carcinoma in adenoma; positive, immunostaining is positive; negative, immunostaining is negative.
* Dysplasia: dysplasia of adenoma; mild, adenoma with mild dysplasia; moderate, adenoma with moderate dysplasia.
* Immunostaining, immunohistochemical staining of MW-fixed tissue: –, negative; +, more than one-half of the tumor cells were positive. Tumors which showed heterogeneous staining were classified on the basis of the immunoreactivity as A2, A2 or T1, T2; A, adenoma; T, focal carcinoma.

RESULTS

Immunohistochemical Analysis of p53 Protein. The tissues from 36 cases included 36 normal mucosae, 37 adenomas (15 adenomas with mild dysplasia and 22 adenomas with moderate dysplasia), and 38 focal carcinomas, as shown in Table 1. Staining of the p53 protein was localized in the nuclei, and no cytoplasmic staining was observed. Nuclear p53 staining was clearly seen in MW-fixed paraffin-embedded sections. Positive staining for the p53 protein was detected in 61% of all the cases of carcinoma in adenoma of the colon. In detail, p53 was detected in only one adenoma with mild dysplasia, 9% of the adenomas.

Table 2 p53 immunopositivity, mutation, LOH, and histopathology in carcinoma in adenoma of colon

<table>
<thead>
<tr>
<th>Histopathological type</th>
<th>Mild dysplasia</th>
<th>Moderate dysplasia</th>
<th>Focal carcinoma</th>
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</thead>
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<tr>
<td>Immunopositivity/total tumors (%)</td>
<td>1/15 (7%)</td>
<td>2/22 (9%)</td>
<td>20/38 (53%)</td>
</tr>
<tr>
<td>Mutation/total tumors (%)</td>
<td>1/15 (7%)</td>
<td>3/22 (14%)</td>
<td>16/38 (42%)</td>
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<tr>
<td>LOH/informative tumors (%)</td>
<td>0/6 (0%)</td>
<td>1/8 (13%)</td>
<td>6/15 (40%)</td>
</tr>
<tr>
<td>Immunopositivity, mutation or LOH/total tumors (%)</td>
<td>1/15 (7%)</td>
<td>4/22 (18%)</td>
<td>25/38 (66%)</td>
</tr>
</tbody>
</table>

* Tumors were classified into three types on the basis of the histopathological diagnosis within the same case.
Fig. 2. Heterogeneity of p53 immunostaining and mutation detected by PCR-SSCP in cases 26 (a-c) and 54 (d-f). In case 26, p53-positive staining is limited to only a few glands of adenoma. a, p53-positive adenoma cells (A2). b, focal carcinoma (T) is uniformly negative. In case 54, heterogeneous staining of focal carcinoma is observed. d, p53-negative carcinoma cells (T1). e, p53-positive carcinoma cells (T2). × 33. c and f, good correlation between p53 immunostaining and mutation is seen in each case. Arrow, the shifted band of mobility.

Mutation and LOH in p53 Gene. Exons 5, 6, 7, and 8 of the p53 gene were examined for mutations by PCR-SSCP. PCR-SSCP was performed at least twice for each sample, and the reproducibility of the result was confirmed. LOH in the p53 gene was examined by PCR-restriction fragment length polymorphism, and it was also confirmed by PCR-SSCP in cases with a mutation and by the sequence of the mixed DNAs from pooled PCR clones in some cases. Fourteen of the 36 cases (39%) showed heterozygosity in the BamHI site and were thus considered to be informative for LOH analysis. The frequencies of p53 gene mutations and LOH in each histopathological type of carcinoma in adenoma are summarized in Table 2. Seven % (one of (two of 22) of adenomas with moderate dysplasia; and 53% (20 of 38) of focal carcinomas (Table 2). As shown in Fig. 1a, practically all of the carcinoma cells in the section were found to be positive for immunostaining in the p53-positive specimens except for two cases (nos. 43 and 54), each of which was composed of two different subpopulations, i.e., positively stained and negatively stained subpopulations (Fig. 2, d and e). In contrast, positive staining for the p53 protein in the adenoma cells of cases 46, 49, and 26 was limited to only a few glands which were surrounded by negative glands (Fig. 2a). Heterogeneity of p53 immunostaining was observed only in these cases (Table 1). The normal mucosa adjacent to the adenoma or carcinoma was completely negative for p53; thus, the expression level of wild-type p53 protein in the normal mucosa was thought to be below the sensitivity of immunohistochemistry.
15) of adenomas with mild dysplasia, 14% (three of 22) of adenomas with moderate dysplasia, and 42% (16 of 38) of focal carcinomas showed abnormally shifted bands in the SSCP analysis. Of the 14 informative cases, none of the adenomas with mild dysplasia, only one of the adenomas with moderate dysplasia, and 40% (six of 15) of the focal carcinomas showed LOH when compared with the normal mucosae. Neither a mutation nor LOH was detected in the normal mucosae. In agreement with the results of immunohistochemical analysis, mutation and LOH in the p53 gene were detected at a low frequency in adenoma and at a high frequency in focal carcinoma.

**Correlation between p53 Immunostaining and Genetic Changes in Carcinoma in Adenoma of Colon.** To understand the correlation between p53 expression and genetic alterations, Table 1 shows a list of individual tumors which were divided into two groups on the basis of the immunoreactivity with PAb1801. Seventy-eight % (18 of 23) of the tumors with positive staining for p53 protein showed a mutation or LOH. Interestingly, in five cases which showed heterogeneous staining in adenoma or focal carcinoma, a mutation was detected only in the glands which stained positively (Fig. 2). Eighty-seven % (45 of 52) with negative staining showed no genetic alteration, whereas 13% (7 of 52) with negative staining showed a mutation or LOH. Interestingly, all of these mutations clustered in exon 6. For these tumors, we analyzed the sequence of exon 6 to identify the type of mutation. Four were nonsense mutations in codon 213 (Fig. 3), and one was a silent mutation in codon 191. In addition, we analyzed the sequence of exons 6 and 8 in four representative tumors which had stained positively and showed mutations. As shown in Fig. 1 (representative data), all of these mutations were missense mutations.

**p53 Gene Alterations from Adenoma to Focal Carcinoma.** To assess the genetic change during the conversion from adenoma to focal carcinoma, we investigated the mutation within the same case, as shown in Table 3. In this table, we judged that a tumor which was positive for either immunostaining or SSCP was a mutant type. Arrows show the tumor development within the same case. Cases 18 and 28 were excluded from this table because adenoma was not available.

**DISCUSSION**

To our knowledge, this is the first extensive study of p53 gene alteration in *carcinoma in adenoma*, which is thought to be a very early phase of malignancy in the colon. Good correlation was found between p53 immunostaining and the presence of genetic alterations (Table 1). Of those tumors which reacted with PAb1801, 78% showed a genetic alteration. There were five focal carcinomas which stained positively with PAb1801 but did not contain a detectable genetic alteration. In those cases, it may be possible that the mutation occurred in a region of the p53 gene other than exons 5–8. A more probable explanation is insufficient sensitivity of the SSCP analysis, as noted previously (19). Thirteen % (7 of 52) of the tumors which were negative for p53 immunostaining showed a mutation or LOH. It has been suggested that some mutations are overlooked by immunohistochemistry using PAb1801 alone (10). For example, a silent mutation in codon 191 in case 31 created a normal protein in the same way of the polymorphism in codon 213 (42). There were four tumors with a nonsense mutation in codon 213 in the present study (Table 1). In addition, we detected a nonsense mutation in codon 196 in three cases of advanced colon carcinoma with negative staining for p53 protein (data not shown). These results support the previous report that nonsense mutations frequently cluster between codons 196 and 213 of exon 6 (43). All of these nonsense mutations lead to a truncated protein. A frame-shift mutation which gives rise to a stop codon may also create a truncated protein. Because PAb1801 recognizes an epitope between amino acids 32 and 79, even the truncated protein should contain the epitopic region. It was suggested that point mutations of the p53 gene produce significant conformational changes in the protein (44–46) and also that PAb240 recognizes an epitope between amino acids 156 and 335 of various mutant proteins due to a common conformational effect (47). Different mutations which create a stop codon may exert a common conformational effect on the epitope of PAb1801, with the result that it would not be recognized. The carboxyl terminus possesses DNA binding activity, as well as the oligomerization domain and the site of nuclear transport (48). Due to the truncation of the carboxyl terminus of the protein, the mutant protein might lose stability and not be detected by immunohistochemistry using PAb1801. Several papers have suggested that a high level of p53 expression correlates with a missense mutation in colorectal carcinoma and several cancer cell lines (11, 13, 15, 16, 19) or with LOH in colorectal carcinoma (17, 18). Considering the conformational effect of different mutations on the PAb1801 epitope, it may be
said that a high level of p53 protein correlates mainly with the presence of a mutation. Because allelic loss of p53 was usually observed together with mutations in invasive colorectal carcinomas (7), it may correlate with LOH. As shown in Table 1, four tumors showed mutation but no LOH. Interestingly, the PAbl1801 staining intensity (not the staining frequency) in these tumors was weaker than that in tumors with both a mutation and LOH. The relatively weak staining seen in the samples without LOH might be due to interactions of the wild-type and mutant p53 proteins.

Many papers have discussed the role of the p53 gene in carcinogenesis of the colorectum (16–18, 24, 28). Those studies have suggested that alterations of the p53 gene are involved in a relatively late stage of colon carcinogenesis because mutation and LOH are common events in invasive carcinoma but rare in adenoma. Moreover, a recent report by Kikuchi-Yanoshita et al. (19) suggested that genetic changes in the p53 gene are associated with conversion from adenoma to early carcinoma. As shown in Table 2, mutations and LOH in the p53 gene were detected at a low frequency in adenoma and at a high frequency in focal carcinoma. These data support their proposal; however, in our series, the frequencies of mutation and LOH in focal carcinoma were very high, and they were comparable to the frequencies in invasive carcinoma of their report. This difference may be due to the high sensitivity of our method of microanalysis coupled with immunohistochemical analysis to obtain relatively pure cell populations. We have already reported that p53 immunopositivity in colorectal carcinoma was not affected by the depth of tumor invasion (35). The immunopositivity of focal carcinoma in this study was comparable to that of invasive carcinoma. These results clearly indicate that alterations of p53 have already occurred in an early stage of carcinoma and that they are associated with malignant transformation, which is the conversion from adenoma to focal carcinoma, rather than with progression from focal carcinoma to invasive carcinoma in the colorectum. Table 3 shows the genetic changes of p53 within the same case. Seventy-one % of the cases acquired alterations of p53 during the conversion from adenoma to focal carcinoma, which strongly supports the concept that adenoma will transform into focal carcinoma if it acquires alterations of the p53 gene.

Studies on the clonality of colorectal tumors have demonstrated that both adenoma and carcinoma have a monoclonal composition in contrast to the normal colonic mucosa, which is polyclonal (49). A recent study on genetic analysis of the c-K-ras locus reported that some adenomas may be heterogeneous but most carcinomas appear to be homogeneous (50). In the present study, three adenomas (cases 46, 49, and 26) showed heterogeneous staining which correlated with the presence of a mutation (Fig. 2, a-c). Each of these adenomas was composed of focally stained glands surrounded by negatively stained glands, both of which were of the same histological grade and morphologically indistinguishable. Moreover, the focal carcinomas in these cases stained negatively. These data seem to contradict the idea that genetic changes present in a certain cell population could provide them with a growth advantage compared with adjacent cell groups (49, 51). However, it has been suggested that nearly all mutant cells are eliminated through the evolution of tumor cell populations because of a metabolic disadvantage or immunological destruction (52). We could not determine whether the mutant cell subpopulations of adenoma would be eliminated or grow into a second carcinoma. It may be speculated that, in these cases, alterations of the p53 gene were not involved in the carcinogenesis. Such cases should be analyzed for other causes of carcinogenesis. The pattern of p53 immunostaining in these colorectal adenomas was not uniform, in accordance with a previous report (53). Two focal carcinomas (cases 43 and 26) showed heterogeneous staining which correlated with the presence of a mutation (Fig. 2, d,f). We revealed in these cases that alterations of the p53 gene are sometimes heterogeneous even in focal carcinoma. In the present study, DNA was extracted from microtissues which were composed of relatively pure cell populations with respect to morphological appearance and immunoreactivity with PAbl1801. Micro-genetic analysis coupled with immunohistochemical analysis is considered to be a very useful method for analyzing the early stage of p53 alterations.

In conclusion, we demonstrated that alterations of the p53 gene occur mainly during the conversion from adenoma to focal carcinoma, which represents the critical step in the adenoma–carcinoma sequence of colon carcinogenesis. Further studies are required to clarify the functional role of the p53 gene in the malignant transformation of adenoma cells and to identify gene(s) other than the p53 gene that are responsible for this step in colon carcinogenesis.

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