Genetic Changes of Both p53 Alleles Associated with the Conversion from Colorectal Adenoma to Early Carcinoma in Familial Adenomatous Polyposis and Non-Familial Adenomatous Polyposis Patients

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ABSTRACT

Mutation and loss of heterozygosity (LOH) in the p53 gene were analyzed in 274 colorectal tumors of 4 histopathological grades. Among 160 tumors from 40 familial adenomatous polyposis patients, none of 58 adenomas with moderate dysplasia had p53 mutations, whereas 8% (3 of 37) of severe adenomas, 15% (6 of 40) of intramucosal carcinomas, and 40% (10 of 25) of invasive carcinomas had p53 mutations. Only 3% (1 of 33) of severe adenomas showed both mutation and LOH, while 25% (6 of 24) of intramucosal carcinomas and 40% (10 of 25) of invasive carcinomas had both mutation and LOH. All intramucosal and invasive carcinomas that had mutations lost the other allele of the p53 gene. In 114 tumors from 86 non-familial adenomatous polyposis patients, similar results were obtained; no adenoma showed both mutation and LOH, but both alterations occurred in intramucosal and invasive carcinomas.

As regards specificity in 56 mutations detected in the present study, the frequently affected codons were codons 175, 238, 245, 248, 273, and 282, 4 of these amino acids being arginine, and 72% (39 of 54) of all mutations were GC to AT transition. Although expression into p53 polyadenylated RNA was high in every invasive carcinoma irrespective of the presence of mutation or LOH, there was a correlation between mutation and protein level; immunostaining of p53 protein was negative in almost all adenomas, but it was positive in 86% of invasive carcinomas exhibiting p53 mutation.

These data suggest that genetic changes on both alleles of the p53 gene through mutation and LOH, which result in abnormal protein accumulation, are involved in the conversion of adenoma to early carcinoma. Also, carcinoma cells with p53 mutations existing within adenoma tissues are detectable by immunostaining, even in formalin-fixed, paraffin-embedded specimens.

INTRODUCTION

The accumulation of alterations in multiple genes appeared to bring about the development of tumors via multiple steps. Previously, we showed that certain genetic changes occurred during carcinogenesis in the colon of patients with FAP, an autosomal dominant disease with a high risk of cancer (1-3). In FAP, it is widely accepted that carcinomas mainly develop from adenomatous polyps through many transition stages of malignancy. We detected LOH on chromosomes 5q, 17p, 18q, and 22q that occurred gradually in the course of the malignant progression; no LOH was observed in adenomas with moderate dysplasia; LOH on 5q and 17p was seen in intramucosal carcinomas; and LOH on 5q, 17p, 18q, and 22q was detected in invasive carcinomas. LOHs on these chromosomes have also been reported in sporadic colorectal tumors (4-9). These data suggest that inactivation of multiple tumor suppressor genes results in the development of tumors. LOH on 17p have been detected in diverse types of carcinomas, which indicates the importance of 17p LOH in carcinogenesis. The target of LOH on 17p have been assumed to be the p53 gene, which is located within the region of loss (10). In addition, mutations in the p53 gene have been observed frequently in various human cancers including colon, breast, lung, liver, brain, ovary, esophagus, bladder, several sarcomas, and leukemias (11-20). Mutation in the p53 gene, therefore, may also have a great effect on the formation of carcinomas. Previous studies have suggested that both allelic loss and mutation in the p53 gene are involved in carcinogenesis of various tissues (12, 20).

Our previous study suggested that LOH on chromosome 17p plays an important role in the conversion of severe adenoma into early-stage carcinoma. To clarify the relationship between the 17p LOH and p53 gene, we analyzed mutations in the p53 gene in 274 colorectal tumors with distinct histopathological grades from both FAP and non-FAP cases, using PCR-SSCP and direct sequencing methods. We detected 56 mutations and found that intramucosal carcinomas showed both 17p LOH and p53 mutation. Several characteristics of p53 mutations were revealed and it was also found that there is a significant correlation between p53 mutation and the level of p53 protein.

MATERIALS AND METHODS

Tumor Specimens. In this study, 160 tumors from 40 FAP patients and 114 tumors from 86 non-FAP patients were analyzed. A part of each specimen was fixed with formalin for diagnosis by histopathological staining and for immunohistochemical staining. The remaining portion was used for analyses of DNA, poly(A) RNA, and protein. These tumor specimens contained more than 70% of tumor tissues.

Histopathological Diagnosis. The specimens were fixed with 10-17% neutral formalin, successively embedded in paraffin, and histologically diagnosed as described previously (3). Adenomas were divided into two classes: adenoma with moderate dysplasia; and adenoma with severe dysplasia. The samples of intramucosal carcinomas which were limited to the lamina propria, including those of adenoma in carcinoma. The term "invasive carcinoma" means strictly a cancer that has invaded through the muscularis mucosae and into the submucosa, muscularis propria, or serosa, according to the General Rules of the Japanese Research Society for Cancer of Colon and Rectum (21). Preparation of Genomic DNA. High molecular weight DNA was extracted from each tumor specimen by treatment with sodium dodecyl sulfate-Pronase K and phenol-chloroform. Also, from normal colorectal mucosa corresponding to each tumor sample, genomic DNA was prepared by the same method.
PCR-SSCP Analysis. The highly conserved regions of the p53 gene, exons 5, 6, 7, and 8, were amplified separately by PCR and mutation was analyzed by the SSCP method (22). The standard reaction mixture (5 μl) consisted of 15.4 μM concentrations of each deoxynucleotide triphosphate, [α-32P]dCTP, the proper pair of each 0.2 μM primer, 1X PCR buffer, Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 50 ng of genomic DNA. The thermal cycles were 3 cycles of 1 min at 97°C, 1 min at 58°C, and 2 min at 72°C and 50 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. The reaction mixture was diluted 100-fold with formamide-dye solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). A 2.2-μl sample of the diluted reaction mixture was heated for 5 min at 80°C and subjected to electrophoresis in 5% polyacrylamide gel containing 5% glycerol. After electrophoresis at 20–25°C, gel was exposed to X-ray film at −70°C. PCR was performed at least twice for each sample and only the reproducible cases were taken. Synthetic oligonucleotides used as primers were

Exon 5 (sense): 5′TGATGAAATCTCCTCTCTTCTGAGTAC 3′
Exon 5 (antisense): 5′TGATGAAATCTCGTCTCCTACTGCTAT 3′
Exon 6 (sense): 5′CAGCTGAGCTCTTATCGTC 3′
Exon 6 (antisense): 5′ATGACAAACCGAGACATCAG 3′
Exon 7 (sense): 5′TGATGAAATCTCGTCTCCTACTGCTAT 3′
Exon 7 (antisense): 5′TGATGAAATCTCCTCTTCTGAGTAC 3′
Exon 8 (sense): 5′TGATGAAATCTCGTCTCCTACTGCTAT 3′
Exon 8 (antisense): 5′TGATGAAATCTCCTCTTCTGAGTAC 3′

Direct Sequencing of the Mutated Strand. From a corresponding gel on PCR-SSCP, an abnormal single-stranded DNA fragment was eluted with distilled water as described (23). The DNA fragment was amplified through the asymmetrical PCR (24) in 100 μl mixture under the same conditions as those for PCR-SSCP analysis, with the exception that the ratio of primers was 100/1 or 1/100 for sense and antisense primers. The asymmetrical PCR was performed twice for each DNA fragment eluted from the gel. The amplified DNA was purified using a QIAGEN-spin20 column (QIAGEN, Inc., Chatsworth, CA), precipitated by isopropanol, and resuspended in 10 μM Tris-HCl (pH 8.5)-1 mM EDTA. The purified DNA was subjected to a dideoxy chain-termination reaction using Sequenase Version 2.0 (United States Biochemical Corporations, Cleveland, OH). Primers used for sequencing were the same as those in PCR-SSCP. These primers were prelabeled with [γ-32P]ATP. The reaction mixture was applied to denaturing electrophoresis in 6% polyacrylamide containing 7 M urea, and the gel was exposed to X-ray film.

Southern Blot Analysis. Each genomic DNA from tumor and its corresponding normal tissue were enzymatically digested, and hybridized with 32P-labeled probe, D17S30 or hp53B, as described previously (3). Loss of heterozygosity was analyzed by comparing the bands of tumor DNA with those of the corresponding normal tissue.

Northern Blot Analysis. Poly(A) RNA was prepared from a tumor specimen using guanidium thiocyanate and oligo(dodecyl)hydroxymidate cellulose. Ten μg of poly(A) RNA were separated electrophoretically in an agarose gel containing formaldehyde and transferred to a nitrocellulose membrane. The membrane was hybridized with 32P-labeled hp53B under the same conditions used for Southern blot analysis.

Western Blot Analysis. Western blot analysis was performed as described (25). Protein was extracted from tumor specimens or cultured colon carcinoma cells with ice cold radioimmunoprecipitation assay buffer. An aliquot of protein (approximately 60 μg) was subjected to electrophoresis in 12.5% sodium dodecyl sulfate-polyacrylamide gel. Separated proteins were transferred electrophoretically to a nitrocellulose membrane. After blocking with 10% skim milk in 100 mM Tris-HCl (pH 7.5)-0.9% (w/v) NaCl-0.1% (v/v) Tween 20, the protein on the membrane was immunologically reacted with PAb1801 (NCL-p53-1801; Novacastra Laboratories, Ltd., Newcastle-upon-Tyne, United Kingdom) and visualized by the standard streptavidin-biotin-alkaline phosphatase method using a Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA).

Immunohistochemistry. For immunohistochemistry, the streptavidin-biotin-alkaline phosphatase method was applied to each specimen. Cultured cells grown on glass coverslips were washed in phosphate-buffered saline, rinsed in acetone for 10 min, and then fixed with 5% buffered formalin for 30 min at 4°C. The cells were then exposed for 30 min at room temperature to anti-human p53 mouse monoclonal antibody PAb1801 (p53-Ab2, Oncogene Science, Inc., Manhasset, NY) at a dilution of 1:500. The formalin-fixed, paraffin-embedded, surgically resected specimens were cut into sections 3 μm thick; deparaffinized in xylene; and rehydrated in a graded ethanol series. The sections were then digested with 0.2% pepsin in 0.1 M HCl and stained using rabbit polyclonal antibody NCL-p53-CM1 (Novocastra) diluted 1:1200 overnight at 4°C. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide in methanol for 20 min. The sections were counterstained with Mayer’s hematoxylin. In all experiments, cases known to be stained positively were included as positive controls. No discrepancy in the results of the immunoreaction between both antibodies was found in the frozen sections of the positive controls (data not shown).

RESULTS

Mutation and Loss of Heterozygosity on p53 in Colorectal Tumors of Distinct Histopathological Type. Tumors were classified into four classes according to histopathological diagnosis. Tumors from FAP patients included 58 adenomas with moderate dysplasia, 37 adenomas with severe dysplasia, 40 intramucosal carcinomas, and 25 invasive carcinomas. Tumors from non-FAP patients included 11 adenomas with moderate dysplasia, 13 adenomas with severe dysplasia, 19 intramucosal carcinomas, and 71 invasive carcinomas.

Mutation in exons 5, 6, 7, and 8 of the p53 gene was examined in these 274 tumors by PCR-SSCP. When the amplified DNA fragments of exon 5, 6, 7, or 8 were separated electrophoretically in nondenaturing polyacrylamide, some single-stranded DNA from tumor specimens showed aberrant mobility distinct from those of normal strands (Fig. 1A). Mutations in these single-stranded DNA were then identified by direct sequencing method after elution of DNA from the gel and amplification of DNA. Every DNA band that showed abnormal mobility had mutation as shown in Fig. 1B. The abnormal bands exhibiting identical mobility with each other on PCR-SSCP had the same mutation. The SSCP pattern of each sample was reproducible.

We detected 56 mutations, and the frequencies of p53 mutation and 17p LOH in colorectal tumors of each histopathological type are summarized in Table 1. In the case of FAP tumors, no adenomas with moderate dysplasia had mutation at least in exons 5 to 8 (0 of 58), whereas 8% (3 of 37) of adenomas with severe dysplasia, 15% (6 of 40) of intramucosal carcinomas, and 40% (10 of 25) of invasive carcinomas had mutation. There was an increase in the frequency that paralleled tumor development. A similar result was obtained regarding the tumors from non-FAP patients. In neither moderate adenomas nor severe adenomas was mutation detected; however, 11% (2 of 19) of intramucosal carcinomas and 49% (35 of 71) of invasive carcinomas contained p53 mutations.

We have previously reported the remarkable increase of LOH on 17p during the conversion of severe adenomas to intramucosal carcinomas (3, 26). In the present investigation, we could detect p53 gene mutation, which seems to be the target of LOH on 17p. The frequency of tumors with both mutation and LOH is also shown in Table 1. Only one adenoma showed both mutation and LOH; however, 25% (6 of 24) of intramucosal carcinomas and 40% (10 of 25) of invasive carcinomas from FAP patients exhibited both mutation and LOH. All of intramucosal and invasive carcinomas containing mutation exhibited LOH. Similar results were obtained in tumors from non-FAP patients (Table 1).
61% (33 of 54) of all point mutations observed, and these hot spots corresponded to other reports on colorectal tumors (19, 20), with the exception that mutations at codons 238, 245, and 282 were not as frequently observed in the published report as in the present study. Mutations detected in the majority of intramusosal carcinomas were at the hot spots.

There were two invasive carcinomas in non-FAP cases that had deletion of bases instead of point mutation. In MY112, 18 bases from the third guanine of codon 174 to the second adenine of codon 180 were deleted, which resulted in the change of amino acid sequence from Arg-Arg-Cys-Pro-His-Glu to Arg. MY115 deleted the last base of codon 157 and all three bases of codon 159, and one base was inserted between codons 155 and 156. These changes resulted in alteration of amino acid sequence from Arg-Val-Arg-Ala (156-159) to Ser-Arg-Arg.

It is also noted that arginine was frequently altered into other amino acids, the frequency being 52% (28 of 54) of all mutations detected. Glycine (codon 245) was the second target amino acid and substituted for valine, serine, or aspartic acid. Cysteine (codon 238) was also affected, which resulted in the loss of thiol residue.

As to the direction of individual base change, the summary is shown in Table 3. Seventy-eight % of all point mutations were transitions, 93% of which were AT to GC. There were 6 tumors (11%) showing G to T transversion, which has never been described in other studies on colorectal tumors.

Expression of p53 Gene in Colorectal Tumors. To understand the nature of genetic changes on the p53 gene in colorectal tumors, we examined the relationship among mutation, LOH, poly(A) RNA level, and protein level.

With respect to expression of the p53 gene into poly(A) RNA, all tumors in the present study showed 2.8-kilobase p53 mRNA, irrespective of the presence of mutation or LOH, although the level of p53 mRNA was much higher in carcinomas than in normal mucosa (Fig. 3A). In the Western blot analysis using monoclonal antibody PAb1801, which recognizes human p53 protein in both normal and mutant form, a remarkable difference was found between tumors with mutation and those without mutation. As shown in Fig. 3B, a p53 protein band was clearly detected in PLK28Ca cells, which had mutation at codon 282, but it was not detected in normal mucosa and PLK73Ca cells which exhibited no mutation in exons 5 to 8. A
similar difference was observed in the immunohistochemical staining of these cells using polyclonal antibody, NCL-p53-CM1 (Fig. 3C). PLK28Ca cells were positive for staining, however, PLK73Ca cells were negative. These results indicate a close correlation between mutation and immunohistochemical staining of p53 protein. In this staining almost all nuclei were stained but cytosol was not stained; furthermore staining was negative in cells at mitotic phase.

Next, we attempted immunohistochemical staining of p53 protein with NCL-p53-CM1 in the formalin-fixed, paraffin-embedded preparations of tumor specimens. The results for each tumor with mutation are shown in Table 2; examples are shown in Fig. 4. Staining was negative in moderate adenoma, e.g., PLK116Ad26 (Fig. 4A), and in almost all severe adenomas. In one severe adenoma, PLK89Ad1 with mutation at codon 244, staining was positive; however, the stained area was limited to the focal grands (Fig. 4B). Intramuscular carcinoma, such as PLK116AdN with mutation at codon 280, was stained in a considerable area (Fig. 4C). Almost all carcinomas with mutation examined in the present study were positively stained, as in the case of MY153, which contained mutation at codon 273 (Fig. 4D). The correlation between p53 mutation and immunostaining in invasive carcinomas is shown in Table 4. In FAP, 8 of 9 (89%) invasive carcinomas representing mutation showed positive staining of p53 protein, whereas in 8 of 10 (80%) those without mutation showed negative staining. A sim-
have never been observed previously in colorectal carcinomas (19, 20). It is possible that there is some difference between hot spots in American and Japanese cases.

Secondly, with regard to direction of base change, transition occurred quite frequently (78%). Most of them (93%) were GC to AT transitions. These directions corresponded to those in previous reports (19, 20) and seem to be common in colorectal carcinomas. Differences have been observed in direction of base changes among tumors of various organs (19). The causes that

DISCUSSION

Our previous report on analyses of LOHs on several chromosomes in correlation with histopathological diagnosis showed that LOH on chromosome 17p did not occur in either moderate or severe adenomas, but it did occur in both intramucosal and invasive carcinomas in FAP patients (3). Thus, it has been suggested that allelic loss on 17p is the key event in the conversion of severe adenoma into an early stage of carcinoma. The significance of 17p LOH has also been postulated in sporadic colorectal carcinogenesis (5, 8). p53, a tumor suppressor gene, is located in the region of this loss on 17p. This gene has been demonstrated to be frequently mutated in diverse human tumors. To investigate the role of LOH on 17p in carcinogenesis, we analyzed mutation and LOH in p53 in colorectal tumors with distinct histopathological grades. In the case of FAP, no adenoma with moderate dysplasia showed mutation, at least in exons 5 to 8 of p53 (Table 1). Mutation in p53 was first detected in severe adenomas, but at low frequency, and in intramucosal and invasive carcinomas with increasing frequency as the carcinoma progressed. The frequency of tumors with both mutation and LOH was very low in adenomas, but it was significantly higher in intramucosal carcinomas and invasive carcinomas. Only one severe adenoma with mutation also showed LOH on p53, whereas all intramucosal and invasive carcinomas with mutation exhibited LOH. Similar results were obtained from tumors of non-FAP cases (Table 1). Therefore, we concluded that both mutation and LOH in p53, i.e., genetic changes in both alleles of p53, were essential in the conversion of severe adenomas into early carcinomas.

Regarding the specificity of p53 mutation, some remarkable features were observed. Firstly, there were 6 hot spots. Sixty-one % (33 of 54) of point mutations detected in this study occurred at six limited codons: codons 175, 238, 245, 248, 273, and 282 (Fig. 2). Of these frequently mutated codons, codons 175, 248, and 273 were in agreement with other reports (19, 20); however, frequent mutation at codons 238, 245, and 282 were newly found in this study. The mutations at codon 245
brought about this specific GC to AT transition in colorectal carcinomas remain to be resolved. Some investigators have pointed out that codons 175, 245, 248, 273, and 282 were all CpG dinucleotide and that 5-methylcytosine formed in CpG was spontaneously deaminated to result in a thymine (27). However, this theory still does not explain how the difference in the tissue specificity of mutations is brought about. Other possible factors are specific mutagens, such as alkylating agents. Treatment of synthetic DNA polymers with \( N^\prime\)-methyl-\( N^\prime\)-nitro-\( N^\prime\)-nitrosoguanidine or methyl methanesulfonate caused GC to AT transition at high frequency (28).

There were some cases where differences in the specificity of mutation among tissues were exhibited. In the K-ras gene, the GC to AT transition was predominant in colon, whereas the GC to TA transversion mainly occurred in lung (29). G to T transversion at codon 249 has been detected frequently in hepatocellular carcinomas from patients exposed to aflatoxin B1 (17, 18), while the mutation at the same codon was A to G transition in a colorectal carcinoma, MY53. Moreover, the frequency of transversion was rather high in hepatocellular carcinoma compared to that in colorectal carcinoma.\(^4\) Therefore, different kinds of carcinogens may act among colon, lung, and liver. The factor that causes allelic loss should also be considered, but this has not yet been elucidated. It is possible that these agents affect the occurrence of mutation directly or indirectly.

A correlation was found between mutation and the level of p53 protein (Fig. 3). It has been suggested that mutant p53 protein is more stable than wild type (30); thus mutant protein has often been detected by immunohistochemical staining, immunoprecipitation, or Western blotting (31, 32). We confirmed that p53 protein, which existed in invasive carcinoma cells with mutation, were obviously stained both in Western blot and in immunostaining using anti-p53 antibody specific to human p53 in both wild and mutant form (33). Taking this into account, we conclude that mutant p53 protein was accumulated in carcinoma cells. No correlation between the expression of p53 into mRNA and mutation suggested that there is no difference in the transcription mechanisms between wild and mutant p53. Immunohistochemical staining of cells was negative at mitotic phase but positive in the nucleus at other phase, as in PLK28Ca cells (Fig. 3), which suggests that expression of p53 protein is regulated during the cell cycle as described (34).

\(^4\) M. Konishi et al., unpublished data.

Table 4    Mutation and immunohistochemical staining of p53 protein in invasive carcinomas

<table>
<thead>
<tr>
<th>Carcinomas</th>
<th>Carcinomas positive for staining (%)</th>
<th>Carcinomas negative for staining (%)</th>
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<tr>
<td></td>
<td>FAP</td>
<td>Non-FAP</td>
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<tr>
<td>With mutation</td>
<td></td>
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<tr>
<td>FAP</td>
<td>8/9</td>
<td>19/23</td>
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<tr>
<td>(89)</td>
<td>(83)</td>
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<tr>
<td>Without mutation</td>
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<tr>
<td>FAP</td>
<td>2/10</td>
<td>4/19</td>
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<td>(20)</td>
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Fig. 4 Immunohistochemical staining of p53 protein in the formalin-fixed, paraffin-embedded tumor specimens. Staining was performed with NCL-p53-CM1. 4-A, PLK116Ad26 (adenoma with moderate dysplasia); 4-B, PLK89Ad1 (adenoma with severe dysplasia showing mutation at codon 244 GGC—GTC); 4-C, PLK116AdN (intramucosal carcinoma showing mutation at codon 280 AGA—ATA); 4-D, MY153 (invasive carcinoma showing mutation at codon 273 CGT—CAT). \( \times 300 \).
Similar correlation was found between p53 mutation and immunohistochemical staining in tumor specimens (Fig. 4; Table 4). In adenomas with moderate dysplasia, staining of p53 protein was negative. Almost every carcinoma with mutation showed positive staining, whereas those without mutation were negative, even though they showed LOH. Thus, immunohistochemical staining of tumor tissues might be useful in the detection of carcinoma cells having mutation as suggested (35).

There were some invasive carcinomas that did not show positive staining, despite having mutation. This may be caused by false-negative immunohistochemical staining because of the difficulty with immunological reaction in paraffin-embedded preparations. On the other hand, there were also several invasive carcinomas without mutation in which immunostaining was positive. It is possible that these carcinomas had mutation in other exons that were not analyzed or that certain types of mutation could not be detected by PCR-SSCP.

The frequency of mutation was lower than that of LOH in the present study. This seems to have been caused by the rather low sensitivity in the detection of mutation compared to that of LOH, for several reasons. It may be possible that mutation occurred in other parts of the genome beyond exons 5–8. The sensitivity in PCR-SSCP was not very high, because we performed electrophoresis of SSCP only in polyacrylamide containing 5% glycerol. It has been reported that some mutations were detectable in electrophoresis without glycerol (22). Furthermore, there were carcinomas in which we could detect neither p53 mutation nor LOH on 17p. Such cases should be analyzed for genetic changes thoroughly, not only to detect the alteration in p53 but also to find other causes of carcinogenesis.

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