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Infrequent p53 gene alterations in ulcerative colitis

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Abstract

The purpose of this study was to determine whether point mutations and loss of the p53 gene take place in ulcerative colitis which is histologically negative for dysplasia. DNA was extracted from 13 frozen rectal or colon biopsies and blood samples. Ulcerative colitis was classified histologically as active (10 cases) and inactive (3 cases). Exons 5-8 were amplified by PCR, treated with exonuclease and shrimp alkaline phosphatase and sequenced by the dideoxy chain termination method with the Sequenase Version 2.0 DNA sequencing kit. PCR products of intron 6 and exon 4 were digested with *Msp*I and *Acc*II, respectively, for RFLP analysis. No p53 gene mutation was detected in these cases. The number of informative patients for loss of heterozygosity (LOH) at the p53 intron 6 was high, 11 out of 12 (92%), whereas no LOH was observed. LOH affecting p53 exon 4 was not detected in lesions from 5 of 12 patients (42%). In ulcerative colitis, tumor progression is similar to that in sporadic colon cancer, and other oncogenes and tumor suppressor genes are likely to be mutated before the p53 gene.

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- LOH
- Mutation

Introduction

When Lane and Crawford (1) and Linzer et al. (2) first described p53 protein as a tumor antigen in 1979, they probably never imagined that its gene would become the most important tumor suppressor gene ever studied. It took some time to realize that the p53 gene was a tumor suppressor (3-6), not an oncogene, as was thought at the beginning (7,8). Frequent deletion of the 17p13.1 region (p53 locus) associated with mutation of the remaining allele in a variety of tumors (9), including sporadic colon cancer (6,10) and colon cancer associated with ulcerative colitis (11-14), indicated that wild type p53 was a tumor suppressor gene.

p53 protein functions as a tumor suppressor, arresting the cell cycle in the G1 phase when DNA is damaged (15), inducing the expression of the p21 protein, an inhibitor of Cdk kinase and proliferating-cell nuclear antigen (PCNA) (16,17). The amino-terminal domain of p21 inhibited cyclin-Cdk kinases and the carboxy-terminal domain inhibited PCNA, two different targets essential for cell-cycle progression and DNA replication (16). Thus, damaged DNA would not replicate, providing time for the repair system to act (15). If this system failed, p53 would induce apoptosis (18,19).

The inactivation of tumor suppressor

genes by deletion and mutation of the remaining allele is considered to play an important role in carcinogenesis (20). The multiple step process in colon tumorigenesis requires the mutational activation of an oncogene and the loss of several tumor suppressor genes. According to the genetic model of sporadic colon cancer progression, carcinoma is preceded by precancerous lesions, i.e., small and large adenomas (21). The loss of the p53 gene by mutation and deletion seems to be a marker for the conversion of adenoma to carcinoma (22), since p53 gene alterations occur near the transition from benign to malignant growth (6). In dysplastic and cancerous ulcerative colitis lesions, p53 gene mutations are also frequent, i.e., they occurred in 35 and 90% of cases, respectively, suggesting that p53 gene inactivation in ulcerative colitis-associated neoplasm progression may be an early event, different from sporadic colon cancer (12).

The purpose of this study, therefore, was to determine whether point mutations and loss of the p53 gene take place in ulcerative colitis which is histologically negative for dysplasia.

Material and Methods

Tissue samples

The study was approved by the Scientific Ethics Committee and the Council of the Department. Rectal or colon biopsy specimens and blood samples were obtained from 13 patients with ulcerative colitis documented by endoscopic and histologic findings who entered the study after giving written informed consent (one patient refused venous blood puncture). The histological lesions were classified according to Riddell et al. (23). These patients were symptomatic; ten patients had active disease but were negative for dysplasia, and three patients had inactive colitis (Table 1). ACD (citric acid/sodium citrate/glucose) was used as anticoagulant. Tissue and blood samples were stored at -70°C prior to DNA extraction.

DNA extraction

DNA was extracted from frozen blood and tissues by the phenol-chloroform method according to Sambrook et al. (24). Up to 43 µg genomic DNA was obtained from a small endoscopic biopsy specimen which was sufficient for the study.

Polymerase chain reaction

One microgram or 500 ng of genomic DNA was used as a template in a reaction volume of 50 µl containing 50 pmol of each primer (Table 2), 200 µM of deoxynucleotide triphosphate (dNTP) and 2.5 U of *Taq* DNA polymerase (Perkin Elmer, Branchburg, NJ, USA). PCR was performed in a 2400 GeneAmp PCR system (Perkin Elmer). Exons 5, 6, 7 and 8 of p53 gene were amplified in 40 cycles according to the following schedule: 93°C for 1 min, 57°C for 2 min and 70°C for 2 min. Intron 6 and exon 4 were amplified in 35 cycles according to the following schedule: 94°C for 1 min, 62°C for 50 s and 72°C for 1 min.

Restriction fragment length polymorphism analysis of the p53 gene

PCR products of intron 6 were digested with 60 U of *Msp*I at 37°C overnight (25); moreover, PCR products of exon 4 were digested with 12 U of *Acc*II at 37°C overnight (26). The DNA fragments were separated by electrophoresis on 4% low melting point agarose gel.

Chain-termination DNA sequencing

PCR products of exons 5-8 were treated with a combination of exonuclease I and shrimp alkaline phosphatase (United States Biochemical, Cleveland, OH, USA). Treated

PCR products were sequenced by the dideoxy chain termination method with the T7 Sequenase version 2.0 DNA sequencing kit (United States Biochemical). Radioactive label was incorporated with α-^{35}SdATP (Amersham, Cleveland, OH, USA). Samples were run on 6% gels; dried gels were exposed to Kodak TMG/RA-1 film at room temperature for 2-5 days.

Results

DNA sequencing

Thirteen ulcerative colitis biopsies were analyzed for mutation in the p53 gene. Exons 5-8 were amplified with primers containing the *Eco*RI restriction site for cloning in the pBluescript plasmid. However, direct sequencing of treated PCR products with exonuclease I and shrimp alkaline phosphatase was performed. Direct sequencing was fast, easy and gave excellent results (Figure 1). No p53 gene mutation was detected in any of the cases studied.

PCR-RFLP analysis of the p53 gene

DNA from tissue samples of twelve patients with ulcerative colitis paired with DNA from leukocytes were studied for the detection of p53 gene loss of heterozigosity (LOH). Two polymorphic sites were chosen, *Acc*II in exon 4 and *Msp*I in intron 6. Primers for

Table 1 - Clinical and pathological data.

Patient	Age	Sex	Duration of disease	Histology	Site
1	28	F	1 year	Active	Left colon
2	53	M	1 year and 6 months	Active	Left colon
3	22	M	1 year	Active	Rectum
4	27	M	2 months	Active	Pancolitis
5	25	F	3 years	Active	Pancolitis
6	32	F	3 years	Inactive	Pancolitis
7	50	M	21 years	Active	Pancolitis
8	57	F	3 years	Active	Pancolitis
9	50	F	7 years	Inactive	Rectum/sigmoid
10	44	F	5 years	Active	Rectum
11	40	F	6 years	Active	Rectum
12	24	M	17 years	Inactive	Right colon
13	26	F	10 months	Active	Pancolitis

Table 2 - Primer sets used in PCR, sequencing and PCR-RFLP.

Primer set/ References	Primer sequences	Priming region	Assays
1	5'-GGGCCCAGGGGTCAGCGGCA-3'	Exon 7 antisense	Sequencing
2 (27)	5'-GGAATTCTCCTAGGTTGGCTCTGAC-3'	Exons 7-8	PCR/Sequencing
	5'-GGAATTCCTGCTTGCTTACCTCGCT-3'	Exons 7-8	PCR/Sequencing
3 (27)	5'-GGAATTCCTCTTCCTACAGTACTCC-3'	Exons 5-6	PCR/Sequencing
	5'-GGAATTCAGTTGCAAACCAGACCTCA-3'	Exons 5-6	PCR/Sequencing
4 (25)	5'-AGGTCTGGTTTGCAACTGGG-3'	Intron 6	PCR/RFLP
	5'-GAGGTCAAATAAGCAGCAGG-3'	Intron 6	PCR/RFLP
5 (26)	5'-AATGGATGATTTGATGCTGTCCC-3'	Exon 4	PCR/RFLP
	5'-CGTGCAAGTCACAGACTTGGC-3'	Exon 4	PCR/RFLP

exon 4 span a region of 259 bp, *Acc*II digestion of the amplified fragments identifies a second allele of 160 and 99 bp. LOH affecting the p53 exon 4 locus was not detected in lesions from five of twelve informative patients (42%). *Msp*I identifies a 2-allele polymorphism with bands of 63 + 44 bp and 107 bp. The number of informative patients for LOH at the p53 intron 6 locus was high, eleven of twelve patients (92%), whereas no LOH was observed.

Discussion

There is convincing evidence that patients with ulcerative colitis have a higher incidence of colorectal cancer than the general population. The risk increases with the duration of the disease (23,28), extensive ulcerative colitis (29) and older age at symptom onset (28). Dysplasia has been a histologic marker of malignant transformation and an indicator for colectomy in these patients (23). It is, thus, of great interest to identify markers for those cases that are likely to progress to high-grade dysplasia or adenocarcinoma.

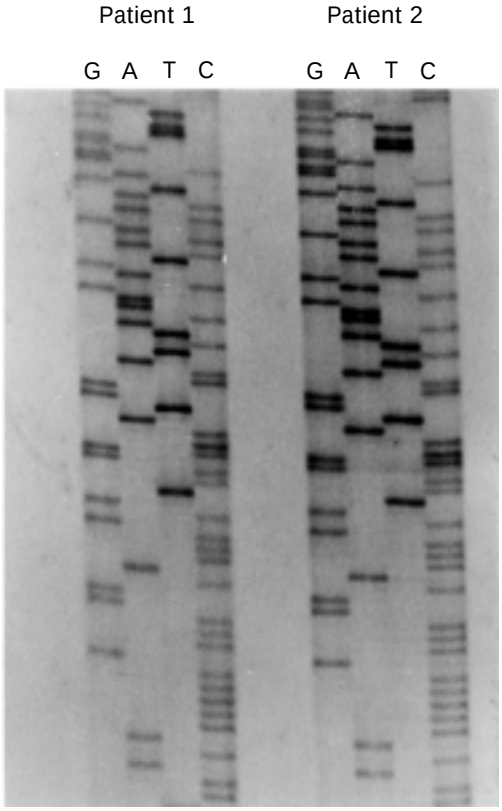

Figure 1 - Wild type p53 exon 5 sequencing from codon 149-TCC to codon 176-TGC in biopsy specimens from patients 1 and 2. The treated PCR product was sequenced by the dideoxy chain termination method with the Sequenase version 2.0 DNA sequencing kit.

We examined p53 gene alterations, allelic deletion and mutation in thirteen symptomatic ulcerative colitis patients negative for dysplasia. Exons 5-8 were amplified and sequenced from DNA of all cases in order to eliminate the possibility of missing a mutation. No p53 gene mutation was detected in these specimens.

The frequency of the loss of heterozygosity in the p53 gene was analyzed by PCR-RFLP. The regions examined were exon 4 and intron 6. Even though the number of informative cases was high, 42% and 92% in exon 4 and intron 6, respectively, LOH was not found in these cases. Our purpose was to observe the inflammatory process itself, since other genetic alterations have been observed at this stage.

We did not observe any p53 gene alteration in ulcerative colitis negative for dysplasia, in agreement with others (30) who did not detect p53 protein overexpression in sections of indefinite dysplasia and no LOH of the p53 gene in epithelium associated with chronic inflammation, except in one case which had dysplasia elsewhere in the specimen (31).

In a recent study on gastric cancer (Mattar R, Alexandrino A and Laudanna AA, unpublished results), we also failed to observe allelic loss of the p53 gene in any of the cases analyzed. Thus, perhaps in our environment the p53 gene is not a frequent target of genetic alterations but other tumor suppressor genes are. This possibility should be considered and verified in a large number of sporadic colon cancer specimens.

Geographical differences in the p53 gene spectrum of mutations were previously reported in hepatocellular carcinoma from sub-Saharan Africa and China (Qidong). In these areas with risk for aflatoxins, a specific mutation of codon 249 of the p53 gene was

found, whereas in other countries, mutations were scattered over four of the five evolutionarily conserved domains, which include codon 249 (32).

Rates of mutation or allelic deletion of the p53 gene were also slightly lower in dysplastic and cancerous ulcerative colitis than in other human cancers (11,13), or they were frequent (12,14) depending on the study referenced. In ulcerative colitis, p53 gene mutation was present in mucosa adjacent to the tumor that was histologically negative for dysplasia or cancer (14), quite different from sporadic colon cancer, where p53 gene mutation is a late event (6).

In conclusion, p53 is not a useful marker of malignant progression in our routine clinical practice since its alterations do not precede dysplasia; other markers should be further evaluated for this purpose.

The activation of the *src* proto-oncogene was reported as being an early event in the genesis of ulcerative colitis colon cancer since *src* tyrosine kinase activity and protein abundance were elevated in neoplastic ulcerative colitis epithelia. However, *src* activity measured in active colitis was remarkably similar to that in normal mucosa or mild quiescent colitis (33).

Another promising marker of malignant progression in ulcerative colitis is the mucin-associated carbohydrate antigen sialosyl-Tn (sTn) that correlates with malignant transformation in sporadic colonic neoplasms. It was expressed in 86% of ulcerative colitis patients who developed cancer in at least one prior nondysplastic surveillance biopsy specimen from the same site (34).

Microsatellites are short nucleotide repeat sequences present throughout the human genome. In a subset of colorectal tumors and other tumors, errors in DNA repair occur, resulting in altered DNA lengths at these regions, a phenomenon which has been termed microsatellite instability. This abnormality occurred at the same frequency in approximately one-fourth of patients with ulcerative colitis-associated dysplasia and carcinoma (35). Recently, microsatellite instability has been detected in 50% of ulcerative colitis patients whose colonic mucosa was negative for dysplasia (36). The authors suggested that the inability of DNA repair mechanisms to compensate for the stress of chronic inflammation might be one mechanism for heightened neoplastic risk in ulcerative colitis.

In ulcerative colitis tumor progression, other oncogenes and tumor suppressor genes are likely to be mutated, preceding the p53 gene as in sporadic colon cancer, or another process may occur in the genetic pathway. The observation that the mismatch repair function is lost due to the inflammatory process in patients with short-term ulcerative colitis (36) and the expression of the antigen sTn in biopsy specimen (34) open a new field of investigation in ulcerative colitis surveillance programs.

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