

# Molecular screening of patients with long standing extensive ulcerative colitis: detection of p53 and Ki-ras mutations by single strand conformation polymorphism analysis and differential hybridisation in colonic lavage fluid

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## Abstract

**Background**—In patients with long standing ulcerative colitis at risk of developing malignancy, mutations of the p53 and Ki-ras gene were investigated in lavage solution obtained at surveillance colonoscopy.

**Methods**—DNA was isolated from 31 consecutive patients with total or subtotal ulcerative colitis and a disease duration of between seven and 26 years. Twenty seven control patients showed no macroscopic or microscopic inflammation on colonoscopy. Exons 5–8 of the p53 gene and exon 1 of the Ki-ras gene were amplified by polymerase chain reaction. Mutations of the p53 gene were detected by single strand conformation polymorphism analysis. Point mutations of the Ki-ras gene were hybridised on dot blots with oligonucleotides marked with digoxigenin.

**Results**—In all cases of ulcerative colitis and in all of the 27 control patients, wild type p53 and wild type Ki-ras could be detected. In four patients with ulcerative colitis, a mutation in exon 5 to 7 of the p53 gene was found, and two patients had a mutation of the Ki-ras gene (Gly to Asp-12, Gly to Val-12). None of these patients had dysplasia in serial biopsy specimens, and all but one had had the disease for more than 10 years. One control patient had a mutation.

**Conclusions**—Mutations were more frequent in patients with long standing ulcerative colitis (19%) than in control patients (3%,  $p = 0.07$ ). The technique may be useful for screening for early malignancy in ulcerative colitis.

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Keywords: p53; K-ras; colonic lavage; ulcerative colitis; molecular screening

One of the major clinical concerns in the treatment of patients with long standing ulcerative colitis is to identify those patients who are at increased risk of malignant transformation. Widely accepted classical risk factors are long disease duration (more than 7–10 years) and involvement of the entire large intestine (pancolitis).<sup>1–3</sup> Repeated colonoscopy with sequential biopsies is recommended as a surveil-

lance procedure. The aim is to detect epithelial cell dysplasia as an early indicator of malignancy, because survival can be prolonged if surgery is performed before the tumour has reached an advanced stage.<sup>4,5</sup> However, the histological grading of dysplasia (mild to severe) has limitations such as interobserver variability, confounding inflammatory changes, and particularly the random nature of biopsies.<sup>5,6</sup>

The identification of genetic alterations characterising malignant transformation is a promising new diagnostic approach. The first successful attempt to identify gene mutations in stool from patients with colorectal carcinoma or adenoma was reported by Sidransky *et al*,<sup>7</sup> followed by several other investigators.<sup>8–16</sup> A major disadvantage of this approach in early colitis carcinoma may be a small tumour load—that is the content of malignant cells in the stool—and impurities acting as polymerase chain reaction (PCR) inhibitors. On the other hand, colonic effluent samples contain less contaminants, can be expected to harbour epithelial cells shed from all sections of the intestine, and provide a convenient source of DNA. Tobi *et al*<sup>13</sup> were able to detect Ki-ras mutations in colonic flushings of patients with a history of colorectal carcinoma, who had no apparent tumour at the time of the investigation. We have shown that colonoscopic lavage solution collected during routine colonoscopy is suitable for the analysis of mutant p53 and Ki-ras in a small series of patients with long standing ulcerative colitis.<sup>16</sup>

The present investigation was carried out to study mutant p53 and Ki-ras in a larger series of patients with long standing extensive colitis and to correlate these findings with disease duration, clinical severity during the course of the illness, and current morphological activity. Control patients without colonic inflammation were similarly investigated.

## Methods

### PATIENT CHARACTERISTICS

Thirty one patients (17 women and 14 men, mean (SD) age 39 (10) years) with a history of total or subtotal ulcerative colitis were studied (table 1). Patients were grouped according to

**Abbreviations used in this paper:** PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

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Table 1 Clinical characteristics of controls and patients with ulcerative colitis for the number of years indicated

	Ulcerative colitis			$\Sigma$	Normal subjects
	7–9 years	10–15 years	> 15 years		
Number	13	10	8	31	27
Age (years)	38 (11)	35 (9)	44 (8)	39 (10)	53 (15)
Sex (F/M)	7/6	6/4	4/4	17/14	15/12
Disease severity					
Mild	2	0	1	3	
Moderate	0	8	5	13	
Severe	11	2	2	15	
Backwash ileitis	1	1	1	3	
Primary sclerosing cholangitis	0	0	0	0	
Haemorrhoids					7
Diverticulosis					6
Irritable colon					3
Normal mucosa					11

Results are mean (SD) at time of colonoscopy.

disease duration (7–9 years, 10–15 years, and more than 15 years), disease “severity”, and morphological activity at the time of colonoscopy. As a measure of disease severity, the drug treatment employed during the course of the disease was assessed. The disease was classified as mild when patients had been treated with salicylates alone (three cases), moderate when corticosteroids were employed (13 cases), and severe when immunosuppressives (usually azathioprine and corticosteroids) were given (15 cases). Current disease activity was assessed semiquantitatively by the endoscopist and the pathologist using conventional criteria. Colonoscopic classification of macroscopic disease was assessed as no significant activity in the presence of mild inflammation with loss of vascular pattern or moderate activity (defined as severe inflammation with contact bleeding), and severe activity (defined as more severe disease with friability, ulcers, or spontaneous bleeding).<sup>17</sup> Pathological grading ranged from mild activity (defined as mild oedema and inflammation) to moderate activity (defined as crypt abscess formation) to severe activity with active ulceration.<sup>17</sup> Three patients presented with backwash ileitis and none had primary sclerosing cholangitis.

Twenty seven control patients (15 women and 12 men, mean age 53 (15) years) showed no macroscopic or microscopic inflammation on colonoscopy (14 had no pathological findings including three with irritable bowel syndrome, seven with haemorrhoids, and six with diverticulosis).

All patients and controls gave informed consent to participate in the study, which was approved by the local ethics committee.

#### PROCESSING OF SAMPLES

The preparation for colonoscopy included a low fibre diet for 24 hours and a laxative taken on the day before and lavage solution taken on the day of colonoscopy until the rectal discharge became clear. All of the lavage solution remaining in the colon (about 300–500 ml) was collected endoscopically in single-use or autoclaved containers. Two biopsy specimens were routinely taken every 10 cm and sent to the pathologist for histological assessment. Two additional specimens were taken at random for DNA analysis.

Lavage fluid was centrifuged at 2700 g; the cell-free supernatant was discarded and the solid material resuspended in phosphate buffered saline. This was repeated three times until the supernatant was clear. The sediment was then stored at  $-20^{\circ}\text{C}$ .

#### DNA ANALYSIS

For DNA extraction a DNA isolation kit (DNA isolation kit USB; Amersham, Braunschweig, Germany) was used and the DNA yield was determined photometrically at 260 nm and 280 nm.

Exon 5–8 of the p53 gene and codon 12 and 13 on exon 1 of the *Ki-ras* oncogene were amplified by PCR using the protocol published previously.<sup>16</sup> Controls were subjected to the same procedure.

Mutations of the p53 gene were analysed by single strand conformation polymorphism (SSCP) analysis of amplified DNA. After non-denaturing polyacrylamide gel electrophoresis, DNA bands were visualised with a 0.1% silver stain and band size was assessed by comparison with known standard DNA (123 bp DNA ladder; Gibco BRL, Bethesda, Maryland, USA) (fig 1). A preparation of wild type DNA from peripheral leucocytes of healthy volunteers served as negative control. As positive controls, DNA was extracted from the cultured epithelial cell line HaCaT with known mutations in exon 5 and 8 on chromosome 17p.<sup>18 19</sup>

For the detection of *Ki-ras* mutations, DNA was blotted on to positively charged nylon membranes (Boehringer, Mannheim, Germany). Identical membranes were hybridised simultaneously with different detection oligonucleotides labelled with digoxigenin (MWG Biotech, Munich, Germany). The signal was detected by chemiluminescence on superimposed x ray film with anti-digoxigenin-alkaline phosphatase Fab fragments. DNA from colorectal carcinoma specimens with known mutations of codon 12 served as positive control.<sup>16</sup> Direct sequencing (both automated and manual) was performed on PCR products of the lavage solution containing a mutation of either the *Ki-ras* or the p53 gene (MWG Biotech).

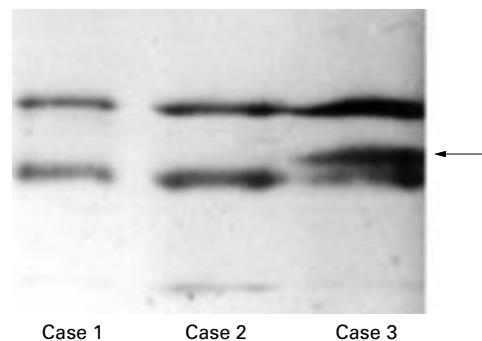


Figure 1 Single strand conformation polymorphism analysis of amplified DNA from three cases. In cases 1 and 2, only wild type p53 is present. In case 3, there is an additional band (arrow), indicating a mutation in the p53 gene.

Table 2 Correlation of p53 and Ki-ras mutations with patient characteristics

	Number of patients	p53 mutations	Ki-ras mutations	Total
Disease duration (years)				
7-9	13	1	0	1/13
10-15	10	3	1	4/10
>15	8	0	1	1/8
Severity of disease				
Mild	3	0	0	0/3
Moderate	13	3	2	5/13
Severe	15	1	0	1/15
Morphological disease activity				
No relevant activity	13	2	1	3/13
Moderate	15	2	1	3/15
Severe	3	0	0	0/3

## STATISTICAL METHODS

Results are expressed as mean (SD). Statistical comparisons between the groups were performed using the Pearson  $\chi^2$  test. Significance was accepted at a  $p < 0.05$  level.

## Results

## p53 AND Ki-ras MUTATIONS IN COLONIC FLUSHINGS

In all samples from patients with ulcerative colitis or control patients, we were able to detect the p53 and wild type Ki-ras genes (figs 1 and 2), whereas all controls without DNA (contamination controls) yielded no signal. SSCP analysis of the p53 gene showed four mutations in exon 5, 6, or 7. Two of the patients with ulcerative colitis had a mutation of the Ki-ras oncogene, one Gly to Asp-12 and one Gly to Val-12 (fig 2, table 2). All detected mutations of the Ki-ras and p53 genes were confirmed by direct sequencing. All but one of the patients had long standing disease of more than 10 years duration (table 2); the one patient with a shorter duration (eight years) and a p53 mutation had severe disease. Three patients had backwash ileitis and one had a Ki-ras mutation. One of the control patients had a mutation of the p53 gene. This patient (haemorrhoids, female, 56 years of age) without macroscopic or microscopic abnormalities in the colon had a family history of pancreatic cancer but no evidence of tumour on thorough examination. She remained healthy for more than 12 months of follow up.

Taken together, 19% of the patients with ulcerative colitis had p53 or Ki-ras mutations as opposed to 3% of the controls ( $p = 0.07$ ).

## HISTOLOGICAL ASSESSMENT OF COLONIC BIOPSY SAMPLES

From the biopsy samples sent to the pathologist, mild dysplasia was detected in four

patients. None of these patients had a mutation in the lavage fluid. In two cases the endoscopist described a suspicious lesion but no dysplasia was found on pathological assessment. None of the patients with a mutation in the lavage had dysplasia in the mucosal biopsy specimens.

## Ki-ras AND p53 MUTATIONS IN COLONIC BIOPSY SPECIMENS

In all cases of dysplasia reported by the pathologist, and in those in which a mutation was found, DNA was extracted from random specimens. A mutation of the Ki-ras gene could not be detected in any of the specimens, although in both those from patients in which a mutation was found in the lavage fluid, sufficient DNA was extracted and the wild type serving as an internal control was detectable. The SSCP analysis of the p53 gene of all four ulcerative colitis samples showed no mutation in the biopsy samples, although sufficient DNA was amplified for SSCP analysis. All samples from cases of dysplasia remained negative when analysed for both p53 and Ki-ras mutations.

## Discussion

In the present study we analysed colonic flushings from 31 patients with long standing ulcerative colitis using molecular screening tools, because mutations may be useful surrogate markers of early neoplasia. The method is based on the observation that cytological specimens collected from the large intestine are not only suitable for cytological assessment<sup>20,21</sup> but also for the analysis of genetic changes using PCR amplification.<sup>11,15,15,16</sup> This approach offers the advantage that cells shed from the entire colon (and probably the small intestine) can be analysed, as mutations have been detected in caecum tumours with the same probability as more distal tumours.<sup>11</sup>

In our series we found mutations of the p53 gene in four patients (13%) and Ki-ras mutations in two (6%). There was no correlation with disease severity or morphological activity in this small series, with an uneven distribution between groups.

However, all patients carrying a mutation, except one, had had the disease for more than 10 years, which is in accordance with clinical experience that long disease duration is one of the major risk factors for the development of carcinoma in these patients.

In our patients, we were unable to correlate mutations found in the lavage with dysplastic

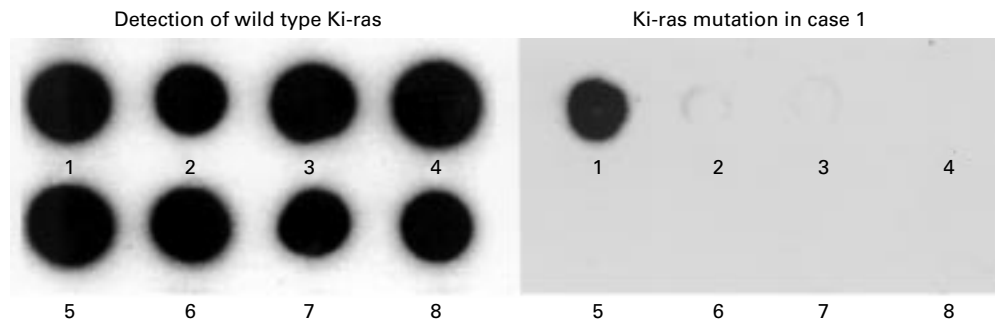


Figure 2 Detection of wild type Ki-ras in cases 1 to 8 (left) and mutated Ki-ras in case 1 (right).

changes observed histopathologically. The fact that no dysplasia was detected in the serial biopsy samples from patients exhibiting mutations in the lavage may be due to the random nature of the biopsies as opposed to a more representative sampling in the lavage. It may also be that a small number of mutant cells may not be recovered in the lavage by our methods.

Primary sclerosing cholangitis<sup>22</sup> and backwash ileitis<sup>23</sup> have been reported as additional risk factors for developing colitis carcinoma. None of our patients had primary sclerosing cholangitis, but three had backwash ileitis. One of the latter patients carried a *Ki-ras* mutation (Gly to Val-12).

Interestingly we were able to detect the wild type *Ki-ras* and p53 in all normal controls, although the amount of cells shed is probably much smaller than in inflammation or neoplasia. In controls we found a mutation in only one case. The clinical meaning of this finding remains unclear, with this patient remaining well after follow up for one year. In the series of Tobi *et al.*,<sup>13</sup> in one patient, effluent was found to harbour a mutant *Ki-ras* allele four years before colorectal cancer was diagnosed.

A review of the literature shows that the risk of malignant transformation in extensive ulcerative colitis begins after 7–10 years and may reach about 10% after 25 years.<sup>24–26</sup> Essentially the same genetic lesions are found in tumours developing in long standing ulcerative colitis as in sporadic colonic carcinoma.<sup>24–26</sup> However, apart from the data presented here, there have only been anecdotal data on the occurrence of mutations in patients with ulcerative colitis but without an apparent tumour. In long standing ulcerative colitis, we cannot be fully content with present cancer screening modalities based on the search for dysplasia.<sup>27</sup> Our data show that it is possible to use colonic lavage fluid for molecular analyses and indicate that with longer disease duration p53 and *Ki-ras* mutations become more frequent.

Therefore it appears reasonable to evaluate this diagnostic tool in a larger number of patients at risk. However, a note of caution is required when molecular screening tools are employed, because there is a false negative rate. In the end, only long term follow up can show whether molecular markers are suitable tools for predicting neoplastic transformation in these patients.

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