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K-*ras* and p53 Mutations in Colonic Lavage Fluid of Patients with Colorectal Neoplasias

M. Heinzlmann S. Neynaber W. Heldwein C. Folwaczny

Department of Gastroenterology, Medizinische Klinik Innenstadt, Ludwig-Maximilians-Universität, München, Germany

Key Words

Gene mutation · Colorectal adenoma · Colorectal carcinoma · Colonic lavage fluid · p53 · K-*ras*

Abstract

Background: The adenoma-carcinoma sequence has its molecular basis in several gene mutations of which K-*ras* and p53 are of paramount importance. The aims of this study were to evaluate whether these genetic alterations can be detected in colonic lavage fluid from patients with colorectal adenomas and carcinomas. ***Methods:*** In 45 patients with adenomas, 20 patients with colorectal carcinomas and 38 patients with non-neoplastic and noninflammatory diseases of the colon p53 and K-*ras* mutations were evaluated in colonic lavage fluid employing single-strand confirmation polymorphism analysis and dot-blot hybridization, respectively. ***Results:*** Mutations of the K-*ras* and the p53 gene were found in 15.6% ($p = 0.065$) of patients with adenomas, in 25.0 % ($p = 0.016$) of patients with carcinomas and in 2.6% in the control group. ***Conclusion:*** Genetic alterations in the colonic lavage fluid could be an additional diagnostic tool for the surveillance of patients with colorectal neoplasias.

Introduction

Colorectal carcinoma is the second most frequent cause of cancer-related deaths in men and women [1]. The incidence rate in Germany is 62/100.000 inhabitants/year. In early stages (Dukes A and B) definite cure is possible, whereas advanced stages (Dukes C and D) are associated with poor prognosis [1, 2]. The mortality rate has not declined substantially during the last 50 years [3]. Present diagnostic and therapeutic strategies aim at a substantial reduction of the mortality by early detection of neoplastic and preneoplastic lesions. Patients with resected adenomas have an increased risk to develop metachronic adenomas or carcinomas [4–6]. In asymptomatic patients, regular fecal occult blood testing (FOBT) decreases the mortality by 23% [7]. The positive predictive value of FOBT for colorectal neoplasms (adenoma and carcinoma) ranges between 22 and 58% [1]. The sensitivity of FOBT in patients with colorectal adenomas is 20–40% [1], and therefore unsatisfactory. Altogether, 40–50% of all colorectal neoplasms are not detected by use of FOBT [8, 9]. Flexible sigmoidoscopy appears to be a more effective screening procedure as it leads to a reduction of 60–80% of deaths related to colorectal cancer [10–12]. However, the acceptance of flexible sigmoidoscopy in asymptomatic patients is low [8, 9]. During endoscopy about 15% of colorectal adenomas might be overlooked, in particular in patients with multiple adenomas [13, 14]. These observations show that current screening procedures are not sufficiently effective or practicable. Due to the drawbacks of the two mainstays in the prevention of colorectal neoplasias, approaches which employ molecular techniques might offer an improvement in the surveillance of patients with an increased risk to develop colorectal carcinomas.

Colonic adenomas are the precusors of colonic carcinomas. The adenoma-carcinoma sequence has its molecular basis in several mutations of tumor-suppressor genes and

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PD Dr. med. Christian Folwaczny
Medizinische Klinik Innenstadt, Ludwig-Maximilians-Universität München
Ziemssenstrasse 1
D–80336 München (Germany)
Tel. +49 89 5160 2270, Fax +49 89 5160 2105

Table 1. Baseline characteristics of the study population

	Female/ male	Age	
		mean ± SD	range
Colorectal adenomas	18/27	69 ± 10	47–91
Colorectal carcinomas	7/13	65 ± 12	86–49
Control	18/20	49 ± 15	20–81

oncogenes, of which K-*ras* and p53 are among the most important [15–18]. The present pilot study aimed to assess the frequency of K-*ras* and p53 mutations in colonic lavage fluid of patients with colonic adenomas and compared it with the frequency of both mutations in controls with non-neoplastic and noninflammatory colonic disorders.

Patients and Methods

Study Population

The study population comprised 45 patients with colorectal adenomas, 20 patients with colorectal carcinomas, 38 controls with non-neoplastic and non-inflammatory diseases of the colon: irritable bowel syndrome (n = 15), hemorrhoids grade I or II (n = 13), diverticulosis (n = 6), perianal lesions, e.g. anal fissures (n = 3) or submucosal leiomyoma (n = 1). The baseline characteristics of the study population are given in table 1. All patients gave informed consent to participate in the study, which was approved by the local ethics committee.

In 22 patients one adenoma, in 8 patients two adenomas, in 6 patients three adenomas and in 5 patients four adenomas were resected. One patient exhibited 5, one patient 6, two patients 8 and one patient 10 adenomas, respectively. In 32 patients adenomas with a diameter of 10 mm ore more were diagnosed. In patients with colorectal carcinomas the distribution was as follows: rectum (n = 7), sigma (n = 8), splenic flexur (n = 1), transverse colon (n = 1) and ascending colon (n = 3).

Patients with carcinomas were staged according to the Dukes classification: Dukes A (n = 3), Dukes B (n = 5), Dukes C (n = 3) and Dukes D (n = 9). Five patients had poorly and 15 patients well-diffentiated adenocarcinomas. Two patients with colorectal carcinomas had additional neoplasias: prostatic carcinoma (pT1c, pN0, pM0, GI) diagnosed 2 years before and carcinoma of the appendix which paralleled the carcinoma in the sigma (Dukes D).

Sampling and Processing of Colonic Lavage Fluid

During routine endoscopy the remaining solution which had been administed orally prior to the endoscopic procedure was aspirated and stored in a sterile vessel. The sample volume was between 50 and 500 ml. Immediately afterwards, the cellular components of the colonic lavage fluid were cleaned by repeated centrifugation and gentle aspiration in PBS solution. Genomic DNA was extracted using a commercially available kit (QIAmp-DNA Mini, Qiagen, Hilden, Germany).

*Detection of K-*ras *and p53 Mutations*

Both procedures have been described in detail elsewhere [19, 20]. Exon 5–8 of the p53-tumor-suppressor gene and codons 12 and 13 of the first exon of the K-*ras* oncogene were amplified employing PCR. p53 mutations were detected by use of single-strand confirmation polymorphism analyses on 13.5% polyacrylamide gels with 10.0% glyerol. K-*ras* point mutations were analysed employing dot-blot hybridization of PCR products on positively charged nylon membranes with different digoxigenin-labelled detection oligonucleotides. The signal was detected by chemiluminescence on superimposed X-ray films with anti-digoxigenin-alkaline phosphatase Fab fragments. DNA extracts from the cultured epithelial cell line HaCaT, with known mutations in exon 5 and 8, codon 179 (C-T) and codon 281 (CC-TT), and tissue specimens of carcinomas carrying the different K-*ras* point mutations in codon 12 and 13 mutations served as positive controls. Preparations of wild-type DNA from peripheral leukocytes of healthy volunteers were used as negative controls. Sequencing was performed on PCR products of DNA of the colonic lavage fluid with a dideoxy-chain-breaking-methode (ABI 377 Sequencer) with specific sequencing PCRs.

Statistical Analysis

The statistical analysis were performed using the two-tailed Fisher's exact test. Significance was accepted at a level of $p < 0.05$.

Results

Mutations of the p53 gene were found in the colonic lavage fluid of 3 patients with adenomas (6.6%) (p = 0.621), in 4 patients with colorectal carcinomas (20.0%) (p = 0.044) and in one control (2.6%). K-*ras* mutations were detected in 4 patients with adenomas (8.8%) (p = 0.121), in one patient with a carcinoma (5.0%) (p = 1.000) but in none of the controls. If the results of both mutations were pooled, 7 patients with adenomas (15.6%) (p = 0.065), 5 patients with carcinomas (25.0%) (p = 0.016) and one patient in the control group (2.6%) displayed genetic alterations in the colonic lavage fluid. p53 and K-*ras* gene mutations were not detected simultaneously. The frequencies of both gene mutations are listed in table 2. The gene mutations in the colonic lavage fluid and the tissue specimens were confirmed by the sequencing of the PCR products.

The one patient in the control group who displayed the p53 mutation (exon 5) in the lavage fluid was a 54-year-old woman. She underwent diagnostic colonoscopy due to a change in bowel habits. The macroscopic appeareance of the colonic mucosa did not reveal any pathologic finding. By extensive clinical evaluation (endoscopy of the upper gastrointestinal tract, barium enema, abdominal sonography, CT scan and X-ray of the thorax) a neoplasm in any other part of the digestive tract, the liver and pancreas was ruled out. Furthermore, during a follow-up of

Table 2. p53 and K-*ras* mutations in the study population

	Samples	p53 mutations		K-*ras* mutations		p53 and K-*ras* mutations	
		n	%	n	%	n	%
Colorectal adenomas	45	3	6.6	4	8.8	7	15.6
Colorectal carcinomas	20	4	20.0	1	5.0	5	25.0
Control	38	1	2.6*	0	0.0	1	2.6

Percent values indicate the percentage of detected mutations within each group.
* For clinical details, see 'Results'.

Table 3. Characteristics of patients with colorectal adenomas and K-*ras* or p53 mutations

Patient	Age years	Sex	Adenomas	Size of adenomas mm	Type of adenomas	Histopathologic classification (dysplasia)	Mutations p53 and K-*ras*	Other neoplasms
S.G.	70	M	10	5–15	tubulovillous	severe	K-*ras:* gly-cys 12	no
P.A.	78	F	8	2–15	tubulovillous	mild	K-*ras:* gly-asp 12	no
M.R.	59	M	1	40	tubulovillous	severe	K-*ras:* gly-val 12	no
S.E.	87	F	3	3	tubular	mild	K-*ras*: gly-asp 13	no
S.B.	72	M	1	5	tubular	mild	p53: exon 6	no
L.H.	79	M	1	8	tubulovillous	mild	p53: exon 6	yes*
V.R.	57	F	1	10	tubulovillous	mild	p53: exon 7	no

* Colonic adenocarcinoma resected 5 years prior to resection of adenomas.

Table 4. Characteristics of patients with colorectal carcinomas and K-*ras* or p53 mutations

Patient	Age years	Sex	Localization of the carcinoma	Type of carcinoma	Differentiation of the carcinoma	Dukes classification	Mutations: p53 and K-*ras*	Other neoplasms
K.J.	82	M	rectum	adenocarcinoma	moderate	D	K-*ras:* gly-asp 13	no
S.J.	59	M	rectum	adenocarcinoma	moderate	A	p53: exon 7	no
B.J.	83	M	rectum	adenocarcinoma	moderate	B	p53: exon 5	no
T.W.	75	M	rectum	adenocarcinoma	poor	C	p53: exon 7	no
S.D.	54	M	rectum	adenocarcinoma	moderate	B	p53: exon 7	no

more than 2 years no evidence for a neoplastic lesion was found. The mother of the patient had died of pancreatic carcinoma at the age of 67 years. Results of genetic analysis of R122H, N29I, and A16V mutations in the cationic trypsinogen gene (PRSS1) (EUROPAC, Liverpool) in the patient's serum, which could be present in patients with hereditary pancreatitis, were negative. Detailed descriptions of patients with colorectal adenomas and carcinomas who exhibited p53 or K-*ras* mutations are given in tables 3 and 4, respectively.

Discussion

The histopathological alterations which can be observed during the progression of colonic adenomas to carcinomas are mirrored by sequential molecular alterations involving several genes, e.g. APC, K-*ras*, DCC and p53 [16–18]. In tissue specimens of sporadic colorectal carcinomas mutations of the tumor-suppressor gene p53 and the oncogene K-*ras* are present in 70 and 50%, respectively [16, 19, 20]. Both mutations play a key role in the adenoma-to-carcinoma sequence. Mutations result in ad-

vanced cellular proliferation and finally lead to aggressive and invasive growth of colorectal carcinomas [21–23]. In large and severely dysplastic adenomas K-*ras* mutations were described more frequently than in smaller adenomas [16]. This observation is compatible with a promoter function of K-*ras* for the development of dysplastic changes and the growth of small colonic adenomas. In vitro, the inactivation of the Ras oncogene results in the loss of the malignant phenotype in colonic carcinoma cell lines [24]. The p53 mutation is possibly the last step in the development of a colorectal carcinoma from an adenoma [16]. Furthermore, the presence of this mutation is a negative prognostic marker for patients with colorectal carcinomas [23, 25, 26].

Sidransky and colleagues [27] found K-*ras* mutations in 8 of 9 stool samples obtained from patients with colorectal carcinomas and presence of K-*ras* mutations in the tumor tissue. Similar results were reported in small series by several other groups [28–34]. In summary, if the respective mutations were present in the tumor tissue, the latter could be detected in about 70% of the stool samples. However, the extraction and amplification of tumor DNA in stool samples is a problematical technical procedure, which might produce false-negative results due to soilings which can impair the amplification of genomic DNA. In contrast, detection of genetic alterations in colonic lavage fluid appears to be a more feasible and validated technique. This has been demonstrated in large patient cohorts [28, 30, 35, 36]. Colonic lavage fluid probably contains cellular elements from all parts of the gastrointestinal tract. It might therefore even be valuable for the detection of gastric, pancreatic oder biliary carcinomas. Furthermore, about 15% of all colorectal adenomas are missed during endoscopy [13, 14]. Hence, this technique might reduce the rate of false-negative results in the surveillance of patients with colorectal neoplasias.

In the present study 15.6% (K-*ras*: 8.8%, p53: 6.6%) of all patients with colorectal adenomas exhibited K-*ras* or p53 mutations in the colonic lavage fluid, as compared to 2.6% in the control group ($p = 0.065$). Furthermore, there was no correlation between the histopathologic degree of dysplasia in the resected adenomas and the detection of genetic alterations in the colonic lavage fluid: only two patients with highly dysplastic adenomas displayed genetic mutations in the colonic lavage fluid. Vice versa five patients with severely dysplastic adenomas exhibited neither K-*ras* nor p53 mutations in the colonic lavage fluid ($p = 0.235$). Presence of large adenomas (≥ 10 mm) [4–6] or multiple adenomas [4, 6] are regarded as important risk factors for the subsequent development of colorectal cancer. There was no correlation with these parameters: in 3 patients with presence of mutations multiple adenomas were diagnosed and in 4 patients, displaying K-*ras* and/or p53 mutations large adenomas were resected. In contrast, in 23 patients who had large adenomas ($p = 1.000$) and in 25 patients with multiple adenomas genetic mutations were not detected ($p = 0.690$).

In our group of patients with colorectal carcinomas gene mutations were present in 25% (p53: 20.0%; K-*ras*: 5.0%). Therefore, the frequency of mutations in the colonic lavage fluid in this study group was significantly higher than in the controls ($p = 0.016$). In patients with gene mutations in the colonic lavage fluid no correlation with the histopathological differentiation of the carcinoma and the Dukes stage was found. One patient had a poorly differentiated adenocarcinoma, whereas the remainder of patients showed a higher degree of histologic differentiation. Only two patients with carcinomas and mutations in the colonic lavage fluid were in an advanced Dukes stage: Dukes C and Dukes D, respectively. Interestingly, all patients with colonic carcinomas exhibiting genetic mutations had carcinomas of the rectum. This correlation has not been demonstrated in previous reports, and might be due to the fact that large amounts of the lavage fluid are usually present in the rectum, as compared to proximal parts of the colon. Thus, we cannot exclude a sampling bias, e.g., epithelial cells from the rectum might be overrepresented in the lavage fluid because of the special anatomic situation with the rectal ampullae. Moreover, this fluid is usually collected at the end of the colonoscopy, which might result in faster processing of DNA origination from the rectum as opposed to DNA from upper parts of the colon.

In summary, due to the low prevalence of genetic alterations in the colonic lavage fluid and the weak correlation with macroscopic or histologic risk factors for the development of colorectal carcinomas this approach does not, at present, offer an advantage compared to standard surveillance strategies of patients with colorectal adenomas. Presence of mutations in the colonic lavage appears to be highly specific for detection of neoplasias. Technical improvements and perhaps additional washings of particular areas might improve the sensitivity of our approach, which is unsatisfactory low, thus for. Moreover, future investigations will aim at the detection of mutations in codon 61 of the K-*ras* gene and further genetic alterations such as the inactivated and mutated APC tumor suppressor gene. This approach could improve the identification of patients with early gate-keeping events. However, long-term follow-up of our patients, an expanded study popula-

tion, the use of further genetic markers and the implementation of reliable DNA purification methods for other body fluids, e.g. stool or plasma [37, 38], might enable physicians to identify patients with a high risk for colorectal cancer on a molecular basis.

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