

Activation of the *ras* genes in malignant and premalignant colorectal tumors

IOANNIS S. GLARAKIS¹, SOTIRIA SAVVA² and DEMETRIOS A. SPANDIDOS¹

¹Laboratory of Virology, Medical School, University of Crete, Heraklion, Crete;

²Department of Pathology, Amalia Fleming Hospital, Athens, Greece

Received September 7, 1998; Accepted September 24, 1998

Abstract. The activation of *ras* genes has been established as one of the steps in the process of colon tumorigenesis. These genes are converted into active oncogenes by point mutations in codons 12, 13, and 61, although a more complex way has also been shown, through alteration of gene expression. In order to investigate the spectrum of *ras* gene mutations, we examined 78 colorectal polypoid adenomas and 76 primary colorectal adenocarcinomas for codon 12 point mutations in K-, H- and N-*ras*, using a PCR-RFLP assay. K-*ras* mutations were found in 42.3% (33/78) of adenomas and in 36.8% (28/76) of carcinomas. Statistically significant association ($p=0.043$) was found between the frequency of K-*ras* mutations in mixed adenomas larger than 2 cm, compared to smaller mixed adenomas. On the other hand, adenocarcinomas harbored more frequently mutations, when indicating development from adenoma ($p=0.016$), in higher grade of differentiation ($p=0.001$) and in females with tumor located proximal to the rectosigmoid ($p=0.013$). No mutations were found in H-*ras*. The incidence of N-*ras* mutations was 1.3% in adenomas and adenocarcinomas (1/78 and 1/76 respectively). Based on our results, we propose the possibility that K-*ras*-dependent tumor development results in the formation of less aggressive neoplasms, than the process of K-*ras*-independent carcinogenesis. Our findings and other previous reports indicate that K-*ras* mutations might be a secondary stress-effect from extrinsic or intrinsic stimulatory factors and that these mutations are not necessarily involved in the malignant transformation of the cell.

Introduction

Tumorigenesis in colon is considered to be a result of the accumulation of multiple genetic alterations in several genes (*APC*, *K-ras*, *p53*, *DCC*, *MCC*) in a single cell (1,2). Point mutations in genes of the *ras* family, mainly in codons 12,

13, and 61, are considered to be the activating mechanism for these oncogenes (3), detected in a variety of human neoplasms (4). This activation might also occur through different pathways, e.g. via the alteration of the expression of the genes (5). Activation of *ras* in colorectal tumorigenesis appears to be an early event (6,7). Mutations of *ras* genes occur frequently in adenomas, which are some of the most common precancerous lesions in carcinogenesis of the large intestine. There are also reports on the presence of K-*ras* mutations in Crohn's disease and ulcerative colitis at a significant frequency, suggesting a possible role of these aberrations in the development of neoplasia (8). Widely varying rates (25-75%) of K-*ras* mutations in colorectal adenomas and sporadic colon carcinomas have been reported (9-12). These differences may be due to the selection methods of the sampling, different methods of mutational analysis, and/or geographic or ethnic differences (6,12-16). The exact activating mechanisms of *ras* genes in human are not yet known. However, colonic bacteria, dietary factors, and bile composition have been reported to relate with colorectal cancer (17-20).

The aim of our study was to investigate the incidence of K-, H- and N-*ras* mutations in precancerous lesions such as polypoid adenomas, and cancerous samples such as primary colorectal adenocarcinomas, to determine the frequency of *ras* activation in a Greek population and to correlate the results with clinicopathological parameters. K-*ras* mutations were more frequent in mixed adenomas larger than 2 cm. Adenocarcinomas carried more frequently mutations when indicating development from adenoma, in females with tumor located proximal to the rectosigmoid, and in higher grade of differentiation.

Materials and methods

Paraffin-embedded tissues from adenomas and adenocarcinomas were obtained from the archives of the Pathology Department of the Amalia Fleming Hospital of Athens, Greece. Seventy-eight polypoid adenomas from 73 patients (a pair of adenomas from 5 patients were available) and 76 sporadic colorectal adenocarcinomas from 75 patients (one patient had 2 different primary foci of cancer) were examined. All adenomas were endoscopically resected, while carcinomas were resected surgically in the period 1991-94. The age range of adenoma group patients was 34-83 years (mean=64.8), and in the adenocarcinoma group 29-87 years (mean=63.9).

Correspondence to: Professor D.A. Spandidos, Laboratory of Virology, Medical School, University of Crete, Heraklion 71409, Crete, Greece

Key words: K-*ras*, H-*ras*, N-*ras*, colon adenomas, colon cancer, PCR-RFLP

The male:female ratio was 47:30 for the adenoma-group (in one case the gender was unknown) and 44:32 for the adenocarcinoma-group.

Five to ten serial sections (5-10 μ m), were cut from each sample, washing the microtome blades with xylene after each block, to avoid contamination of DNA from different samples.

DNA extraction. For DNA extraction, samples were incubated with digestion buffer (containing: 100 mM NaCl, 10 mM Tris-Cl, 25 mM EDTA, 0.5% SDS pH 8.0, 0.1 mg/ml proteinase K), for 2 days at 60°C, followed by 5 days at 37°C. Fresh proteinase K was added every 12 h. After the digestion period, sequential steps of phenol-chloroform were used to inactivate the proteinase K and the DNA was precipitated with ethanol and diluted in ddH₂O.

PCR amplification. Primers used to amplify codon 12 of H-, K- and N-*ras* were those described previously (21,22). PCR analysis was performed in a 50 μ l reaction volume containing 200 ng of genomic DNA, 1 μ M of each primer, 200 μ M dNTPs, 5 μ l of 10x buffer [670 mM Tris-HCl, pH 8.5; 166 mM ammonium sulfate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 μ M β -mercaptoethanol and 1% (w/v) Triton X-100] and 1.25 U of *Taq* DNA polymerase (Gibco BRL). The reactions were denatured for 5 min at 95°C and the DNA was subsequently amplified for 35 cycles at 95°C for 55 sec, 54-62°C (depending on the primers) for 35-45 sec and 72°C for 45 sec each step.

RFLP analysis. K-*ras* and N-*ras*: 10-40 μ l aliquots of the amplification products were digested with 30 U of *Bst*NI at 60°C, overnight. H-*ras*: 10-40 μ l aliquots of the amplification products were digested with 30 U of *Msp*I at 37°C, overnight. Ten μ l of the PCR product was electrophoresed in an 8% polyacrylamide gel and silver stained. All the experiments were repeated at least twice and the results were highly reproducible.

Statistical analysis. To interpret the data Fisher's exact test, or χ^2 test were used, depending on the sample size. In few cases some clinical or histopathological data were not available.

Results

The incidence of point mutations for K-, H- and N-*ras* in colorectal adenomas was 42.3% (33/78), 0% (0/78) and 1.3% (1/78) respectively. Representative results for K-*ras* and N-*ras* mutations using the PCR-RFLP method, described in Materials and methods, are shown in Figs. 1 and 2 respectively. The clinical and histopathological data pertinent to the adenomas were: sex, age, location of the tumor, size, grade of dysplasia, histological type, foci of *in situ* carcinoma and presence or absence of a stalk. K-*ras* mutations were statistically significantly more frequent in mixed adenomas larger than 2 cm, than in smaller mixed adenomas (p=0.043) (Table I). Trends in the incidence of K-*ras* mutations, were shown also for: a) persons older than 64 years (50%), in comparison with younger persons (34%), b) villous and mixed adenomas (50%), in comparison with tubular adenomas (31%), c) high grade of dysplasia (52%), compared with low

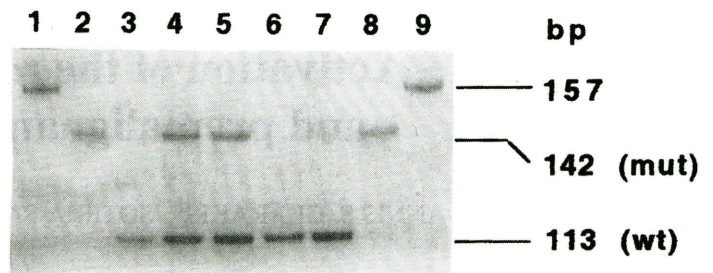


Figure 1. Representative results from K-*ras* analysis from colorectal adenomas. PCR-RFLP analysis revealed two mutant samples in lanes 4 and 5 and normal samples in lanes 3, 6, 7. Lanes 1 and 9, undigested PCR product (157 bp); lanes 2 and 8, digested PCR product from pK12m (homozygously mutated in K-*ras* in codon 12). mut, mutant; wt, wild-type.

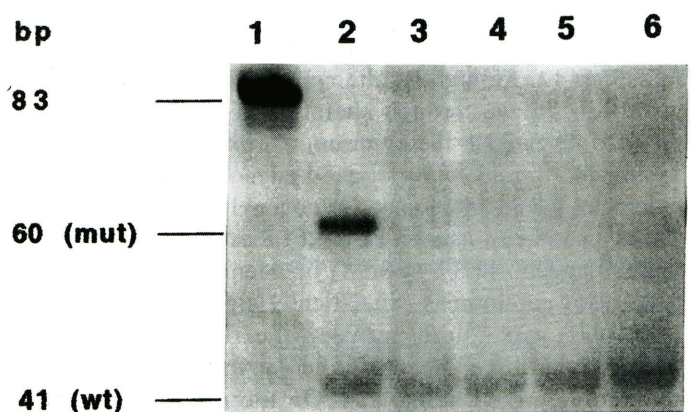


Figure 2. Representative results from N-*ras* analysis from colorectal adenomas. PCR-RFLP analysis revealed a mutant sample in lane 2 and normal samples in lanes 3-6. Lane 1, undigested PCR product. mut, mutant; wt, wild-type.

Table I. Adenomas: the presence of K-*ras* mutations compared to histological type and size of adenomas.

Type and size of the adenomas	K- <i>ras</i> positive samples	K- <i>ras</i> negative samples	% of patients with <i>ras</i> mutation	p-value
Mixed >2 cm	6	1	86	0.043
Mixed \leq 2 cm	13	17	43	

grade (36.5%) and d) adenomas with foci of *in situ* carcinoma (66.5%), in comparison with *in situ* carcinoma-negative adenomas (40%). In 5 cases, two separate adenomas were available for examination from each patient. Of these, 3 cases were negative for K-*ras* mutations, while the other two, both adenomas, were found to carry K-*ras* codon 12 point mutations.

The incidence of K-, H- and N-*ras* codon 12 point mutations in colorectal adenocarcinomas, was 36.8% (28/76), 0% (0/76) and 1.3% (1/76) respectively. Data pertinent to

Table II. Adenocarcinomas: correlations between the presence of *K-ras* and grade of differentiation, location of tumor in females and elements indicating an adenoma-derived tumor.

Clinical or histopathological parameters	<i>K-ras</i> positive samples	<i>K-ras</i> negative samples	% of patients with <i>ras</i> mutation	p-value
Female-rectosigmoid	3	14	18	
Female rest of the colon	9	6	60	0.013
High differentiation	8	1	89	
Moderate and low differentiation	20	47	30	0.001
Tumors with adenoma elements	10	6	63	
Tumors without adenoma elements	18	42	30	0.016

colorectal adenocarcinomas were: sex, age, Dukes' stage, grade of differentiation, mucous production, necrosis in tumor mass, tumor location and elements indicating development from adenoma. *K-ras* mutations were significantly more frequent in (Table II): a) high grade of differentiation in comparison with moderate and low grade ($p=0.001$), b) carcinomas with elements indicating a development from adenoma, in comparison with carcinomas with no such elements ($p=0.016$) and c) females who had the tumor located proximal to the rectosigmoid, compared to rectosigmoid location ($p=0.013$). Although not statistically significant *K-ras* mutations were more frequent in: a) ages below 50 years (71.4%), compared to older persons (34.4%), b) tumors located at the rest of the colon (46.8%), compared to those of rectosigmoid (29.5%) and c) carcinomas with no foci of necrosis in tumor mass (42.8%), compared to those with necrosis (29.5%). One patient had two primary adenocarcinomas in the large intestine, both carried *K-ras* mutations.

Discussion

Ras mutations have been implicated in tumorigenesis, especially in carcinogenesis of the large bowel. Elevated expression of *ras* oncogenes has been reported in polyps and malignant tumors of the large bowel (23). A model has been proposed to explain the adenoma-carcinoma sequence in colorectum (1). Other investigators propose different, *ras*-independent pathways, for some colorectal carcinomas (14,24,25).

In the present study, we detected *K-ras* codon 12 point mutations in 42% (33/78) of adenomas and 36.8% (28/76) of adenocarcinomas. No *H-ras* mutation was detected, while *N-ras* mutations were found in only 1.3% of adenomas and adenocarcinomas (1/78 and 1/76 respectively). The observed difference in the *K-ras* mutation frequency in our study, can be attributed to genetic and environmental factors. Differences in ethnic origin may determine a diverse sensitivity to certain carcinogens. In addition, the environmental background and diet of the studied population is rather different from that of Northern Europe or America. The heterogeneity of the distribution of *K-ras* mutations in adenomas and adenocarcinomas might also lower the rates of mutations detected (11,14,26).

Our results show that *K-ras* mutations are more frequent in females with adenocarcinomas proximal to the rectosigmoid ($p=0.013$), compared to rectosigmoid location. Many environmental and intrinsic factors have been implicated in colorectal tumorigenesis. Dietary factors such as consumption of animal fat (18) and meat (27) are associated with high risk of developing colorectal cancer, while others (fiber, vitamins) are associated with low risk. Considering the fact that bile acids are reabsorbed in the proximal colon and have been implicated in tumorigenesis through bacterial action (17) and that constipation has a higher prevalence in females than in males (28), it could be of interest to correlate these events with the higher incidence of mutations we have found in females who had the tumor located in the proximal colon.

The hypothesis that other precursor genetic alterations, such as damaged mismatch repair genes, have been implicated in hereditary types of colorectal cancer (29) could help to interpret the higher incidence of *K-ras* mutations we found in carcinomas from patients aged less than 50 years; an age range where patients are usually suspect for a hereditary background.

In our findings *K-ras* mutations are more frequent in mixed adenomas larger than 2 cm. This is consistent with reports suggesting that malignant transformation in these types of adenomas is over 45% (30).

We showed that *K-ras* mutations are less frequent in mild and moderate grade of differentiation of carcinomas, which are considered more aggressive. Similar findings come from our studies in carcinomas with foci of necrosis, which show a more extensive growth range. Moreover *K-ras* mutations are less frequent in carcinomas which have no indication of adenoma elements, and this might be due to a fast tumor development and destruction of the adenoma elements. All the above suggest that *K-ras*-dependent tumor development results in the formation of less aggressive neoplasms, than the process of *K-ras*-independent carcinogenesis. This concept is consistent with reports which associate *K-ras* mutations with longer survival (31). *K-ras* mutations have also been found in hyperplastic polyps in high frequency (47%) (32), which are not considered precancerous lesions. In addition, mutations have been found in adenomas containing foci of *in situ* carcinoma, but only in the adenoma region and not in the carcinoma region (33). The above findings imply two

