Activation of the ras genes in malignant and premalignant colorectal tumors

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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).
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 μm), 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 μl reaction volume containing 200 ng of genomic DNA, 1 μM of each primer, 200 μM dNTPs, 5 μl of 10x buffer [670 mM Tris-HCl, pH 8.5; 166 mM ammonium sulfate; 67 mM magnesium chloride; 1.7 mg/ml BSA; 100 μM 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 μl aliquots of the amplification products were digested with 30 U of BanII at 60°C, overnight. H-ras: 10-40 μl aliquots of the amplification products were digested with 30 U of MspI at 37°C, overnight. Ten μl 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 χ² 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 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.

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

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
possibilities: a) that occasionally other genetic pathways, different from the known adenoma-carcinoma sequence lead to carcinogenesis in the colorectum and b) that ras mutations might be a secondary event, possibly caused by extrinsic or intrinsic stimulatory (and not necessarily carcinogenic) factors, or as a result of other changes in DNA and it is not necessarily involved in the malignant transformation of the cell.

Acknowledgements

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References