K-ras mutations and RASSFIA promoter methylation in colorectal cancer

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Human cancer is characterized by genetic and epigenetic alterations. In this study we provide evidence for the interruption of Ras signaling in sporadic colorectal cancer (CRC) by either genetic activation of the K-ras oncogene or genetic silencing of the putative tumor suppressor gene RASSFIA. Paraffin embedded tumor tissue samples from 222 sporadic CRC patients were analysed for K-ras codon 12 and codon 13 activating mutations and RASSFIA promoter hypermethylation. Overall, K-ras mutations were observed in 87 of 222 (39%) and RASSFIA methylation was observed in 45 of 222 (20%) of CRCs. Mutation of K-ras alone was detected in 76 of 222 (34%) CRCs. RASSFIA promoter methylation with wild-type K-ras was observed in 34 of 222 (15%) CRCs. In 101 of 222 (46%) CRCs neither K-ras mutations nor RASSFIA methylation was observed and 11 of 222 (5%) CRCs showed both K-ras mutations and RASSFIA methylation. These data show that the majority of the studied CRCs with K-ras mutations lack RASSFIA promoter methylation, an event which occurs predominantly in K-ras wild-type CRCs (P = 0.023, Chi-square test).

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The Ras family of small GTPases includes three highly similar p21 proteins: H-, K- and N-ras, which are encoded by different genes and which function as molecular switches in cell proliferation, differentiation and apoptosis signal transduction pathways (Campbell et al., 1998; Crespo and Leon, 2000). Ras proteins, which are located at the inner plasma membrane, are activated transiently as response to extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors (Campbell et al., 1998). The biological activity of Ras proteins is mediated by an effector domain, by which these proteins bind to different effector molecules (Campbell et al., 1998; Crespo and Leon, 2000; Shields et al., 2000). The hallmark of Ras function is a transition between an inactive state, in which the proteins are bound to GDPs and an active state in which conversion to GTPs occurs. This transit is governed by two types of regulatory proteins: guanine nucleotide exchange factors which catalyze the GDP/GTP exchange and GTPase-activating proteins which enhance the intrinsic capacity of Ras proteins to hydrolyze GTP into GDP, thereby returning Ras to the inactive state (Crespo and Leon, 2000).

Mutant, activated forms of Ras proteins, which are frequently observed in cancer, have an impaired GTPase activity rendering the protein resistant to inactivation by regulatory GAP proteins (Bos, 1989; Crespo and Leon, 2000). Known activating mutations are mainly found in codons 12 and 13, and to a lesser extent in codons 59, 61 and 63. The frequency of each ras oncogene in tumors varies, depending on the tissue of origin for the neoplasia. H-ras mutations are preferentially found in cancers of the skin and in squamous head and neck tumors, whereas N-ras mutations are common in hematopoietic malignancies. K-ras mutations are mainly detected in adenocarcinomas of the lung, pancreatic carcinomas and CRCs (Rodenhuis, 1992).

Recently a new Ras effector homologue was characterized (Dammann et al., 2000) named RASSF1, which is located at chromosome 3p21.3, a region frequently showing allelic loss in many cancers (Kok et al., 1997). The gene for RASSF1 encodes two major transcripts, RASSF1A and RASSF1C, which are produced by alternative. CpG island containing promoters and alternative mRNA splicing. The C splice variant of RASSF1 binds Ras in a GTP-dependent manner and mediates the apoptotic effects of oncogenic Ras (Vos et al., 2000). RASSF1C expression is reduced in ovarian tumor cell lines (Vos et al., 2000), but not in breast, lung and kidney cancer cell lines (Burbee et al., 2001; Dreijerink et al., 2001). The RASSF1A isoform, which shows homology with the Ras effector Nore 1, has a putative ATM phosphorylation site and binds to the DNA repair protein XPA, which suggests it is involved in DNA repair and cell cycle control (Agathanggelou et al., 2001; Dammann et al., 2000).

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Promoter methylation of \textit{RASSF1A} and subsequent reduction of expression has been recently reported in a very high proportion of small cell lung cancer (Damman et al., 2001a) and nasopharyngeal cancer (Lo et al., 2001) and to a lesser degree in non small cell lung cancers (Dammann et al., 2000), bladder cancer (Lee et al., 2001), breast cancer (Agathanggelou et al., 2001; Burbee et al., 2001; Dammann et al., 2001b), ovarian cancer (Agathanggelou et al., 2001; Yoon et al., 2001), renal cell carcinoma (Dreijerink et al., 2001; Morrissey et al., 2001; Yoon et al., 2001) and gastric cancer (Byun et al., 2001). Although not studied simultaneously in the same tumors, the highest incidence of \textit{RASSF1A} methylation is observed in tumor types with infrequent \textit{ras} gene mutations, i.e. small cell lung cancer and nasopharyngeal cancer (Bos, 1989). This is consistent with observations that simultaneously occurring (epi)genetic alterations of genes in the same signaling pathway are seldomly observed in a single tumor. For example, mutations in the regulatory domain of \textit{\beta}-catenin and APC gene were observed to be mutually exclusive in CRC, consistent with their similar effects on \textit{\beta}-catenin stability and Tcf activation (Sparks et al., 1998). Furthermore, simultaneous inactivation of \textit{Rb} and the p16\textsuperscript{INK4a} locus, which negatively regulates the phosphorylation of Rb, is rarely found in any tumor type (Serrano, 1997).

To test whether this association also holds true for the \textit{Ras} signaling pathway, we examined \textit{K-ras} mutations and \textit{RASSF1A} promoter methylation in sporadic CRC (\textit{n} = 222), a cancer type with a relatively high frequency (40\%) of \textit{K-ras} mutations (Rodenhuis, 1992). We detected \textit{K-ras} mutations in 87 of 222 (39\%) CRCs, a frequency consistent with previous reports on the mutational frequency in primary colon cancer. Examples of \textit{K-ras} sequencing are shown in Figure 1. We did not observe any association of \textit{K-ras} mutation with age at diagnosis, gender, Duke stage or location of the tumor (Table 1).

From our hypothesis that \textit{K-ras} gene mutations and \textit{RASSF1A} methylation are mutually exclusive, it would be predicted that the frequency of \textit{RASSF1A} methylation in primary CRCs would be lower than the frequencies observed in tumor types which rarely show \textit{ras} gene mutations (Bos, 1989; Rodenhuis, 1992), i.e. primary small cell lung cancer (71\%–79\%) (Agathanggelou et al., 2001; Burbee et al., 2001; Dammann et al., 2001a), breast cancer (49\%–62\%) (Burbee et al., 2001; Dammann et al., 2001b), renal cell carcinoma (23\%–91\%) (Dreijerink et al., 2001; Morrissey et al., 2001; Yoon et al., 2001) nasopharyngeal cancer (67\%) (Lo et al., 2001), bladder cancer (35\%–62\%) (Lee et al., 2001; Maruyama et al., 2001) and neuroblastoma (55\%) (Astuti et al., 2001).

To study \textit{RASSF1A} methylation, MSP primers were located in the 5' region of this gene, where methylation has been previously observed and tested by analysing the lung cancer cell line A549 as a methylated control (Dammann et al., 2000). DNA from normal breast tissue (\textit{n} = 4), normal lung tissue (\textit{n} = 4) and normal lymphocytes (\textit{n} = 3) was also examined for methylation changes in this region. The A549 cell line was fully methylated for \textit{RASSF1A}, while DNA from normal lymphocytes, normal breast and normal lung tissue was unmethylated (Figure 2).

In CRC, \textit{RASSF1A} methylation was observed in 45 of 222 (20\%) of the tumors. Examples of the \textit{RASSF1A} MSP are shown in Figure 2a. We also analysed DNA from normal colon tissue obtained from six non-cancer patients. None of the six tissues showed \textit{RASSF1A} methylation (Figure 2b). In addition to tissue from non-cancer patients, DNA from normal colon epithelium microdissected from seven CRCs was analysed. In six of seven cases, normal colon DNA was
Table 1. Clinical and pathological parameters of K-ras wildtype (WT) and mutant (MUT) and RASSFIA unmethylated (U) and methylated (M) CRCs and of the total group of CRCs

<table>
<thead>
<tr>
<th></th>
<th>K-ras WT (n = 135)</th>
<th>K-ras MUT (n = 87)</th>
<th>RASSFIA U (n = 177)</th>
<th>RASSFIA M (n = 45)</th>
<th>Total (n = 222)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (y)</td>
<td>67.7</td>
<td>68.3</td>
<td>67.6</td>
<td>69.1*</td>
<td>67.9</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>80 (59.3%)</td>
<td>49 (56.3%)</td>
<td>100 (56.5%)</td>
<td>29 (64.4%)</td>
<td>129 (58.1%)</td>
</tr>
<tr>
<td>F</td>
<td>55 (40.7%)</td>
<td>38 (43.7%)</td>
<td>77 (43.5%)</td>
<td>16 (35.6%)</td>
<td>93 (41.9%)</td>
</tr>
<tr>
<td>Dukes stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>26 (19.3%)</td>
<td>25 (28.7%)</td>
<td>41 (23.3%)</td>
<td>10 (22.2%)</td>
<td>51 (23.0%)</td>
</tr>
<tr>
<td>B</td>
<td>51 (37.8%)</td>
<td>28 (32.2%)</td>
<td>62 (35.0%)</td>
<td>17 (37.8%)</td>
<td>79 (35.6%)</td>
</tr>
<tr>
<td>C</td>
<td>36 (26.7%)</td>
<td>20 (23.0%)</td>
<td>44 (24.9%)</td>
<td>12 (26.6%)</td>
<td>56 (25.2%)</td>
</tr>
<tr>
<td>D</td>
<td>16 (11.9%)</td>
<td>8 (9.2%)</td>
<td>21 (11.9%)</td>
<td>3 (6.7%)</td>
<td>24 (10.8%)</td>
</tr>
<tr>
<td>UK</td>
<td>6 (4.4%)</td>
<td>6 (6.9%)</td>
<td>9 (5.0%)</td>
<td>3 (6.7%)</td>
<td>12 (5.4%)</td>
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<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Colon</td>
<td>95 (70.4%)</td>
<td>54 (62.1%)</td>
<td>117 (66.1%)</td>
<td>32 (71.1%)</td>
<td>149 (67.1%)</td>
</tr>
<tr>
<td>Rectosigmoid</td>
<td>15 (11.1%)</td>
<td>11 (12.6%)</td>
<td>19 (10.7%)</td>
<td>7 (15.6%)</td>
<td>26 (11.7%)</td>
</tr>
<tr>
<td>Rectum</td>
<td>25 (18.5%)</td>
<td>22 (25.3%)</td>
<td>41 (23.2%)</td>
<td>6 (13.3%)</td>
<td>47 (21.3%)</td>
</tr>
</tbody>
</table>

*P = 0.033 T-test. Paraffin embedded CRC tissue samples were obtained from 222 patients who participate in The Netherlands Cohort Study on Diet and Cancer. The study design has been described in detail elsewhere (van den Brandt et al., 1990). Record linkage covering the period up to the end of 1993 (7.3 years follow-up), and excluding the first two years of follow-up, resulted in 819 incident CRC patients (ICD-O 153.9, 154.0 and 154.1). The distribution of age, gender, Dukes stage and location of tumor of the patients in this study are representative for the 819 eligible CRC patients. The study protocol was approved by the ethics committee of the University Hospital Maastricht. Differences in mean values or in categorical variables between two groups were tested using the t-test and Chi-square test respectively. Data were analyzed using SPSS software (version 9.0).

In concordance, DNA isolated from normal colon tissue showed RASSFIA methylation. This patient also had RASSFIA methylation in DNA obtained from adjacent tumor tissue (Figure 2b). RASSFIA methylation in this 'normal' colon tissue might be explained by the presence of contaminating tumor cells or the presence of a field defect in colon mucosa.

As observed for K-ras mutations, RASSFIA methylation was not associated with gender, Dukes stage and location of the tumor (Table 1). Age at diagnosis was slightly higher (P = 0.033, T test) in RASSFIA methylated CRCs. RASSFIA methylation, however, was largely confined to tumors without K-ras mutations. RASSFIA promoter methylation without K-ras mutations was observed in 34 of 222 (15%) CRCs, while 76 of 222 (34%) CRCs showed K-ras gene mutations without RASSFIA promoter methylation. This difference between methylation of RASSFIA versus K-ras mutations was statistically significant (P = 0.023, two-tailed Chi square test).

Since the majority of mutations in K-ras occur in codons 12 and 13, inclusion of codons 59, 61 and 63 in the K-ras gene analysis would not be expected to increase the prevalence of K-ras mutations in this study significantly. Therefore, our observation that 46% (101 of 222) of the CRCs contain neither K-ras codon 12 and 13 mutations nor RASSFIA methylation, suggest that other genes in the Ras signal transduction pathway may be altered in CRC. Alternatively, Ras signaling may not be affected in all CRCs.

A small proportion (11/222 (5.0%)) of CRCs had both RASSFIA methylation and K-ras mutations. These CRCs did not show specific characteristics concerning type of mutations of clinicopathological parameters. Redundancy in alterations of members of the same pathway is sometimes observed, i.e. p14ARF methylation and p53 mutations (Esteller et al., 2000). One explanation is that the coexistence of alterations

![Methylated and unmethylated RASSFIA MSP reactions](image_url)
of both RASSF1A and K-ras is a rare, stochastic event. Alternatively, this could suggest that methylation of RASSF1A and mutational activation of K-ras are not functionally equivalent.

While the frequency of RASSF1A methylation has been reported for many different types of cancer, little data concerning the function of this gene has been published. Exogenous expression of RASSF1A cell lines lacking expression decreased in vitro colony formation and in vivo tumorigenicity (Burbbee et al., 2001; Dammann et al., 2000). No studies have examined whether RASSF1A inactivation leads to constitutive activation of the Ras signaling pathway. However, our data provide further evidence for a role in cancer in that inactivation of RASSF1A occurs predominantly in CRCs without alteration of K-ras itself, and may provide an alternative pathway for affecting Ras signaling. The factors responsible for whether a cell will exhibit DNA methylation changes, gene mutations or allelic losses need to be clarified, although different exposures to environmental carcinogens might contribute to these differences.

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References