Mutational and Nonmutational Activation of p21ras in Rat Colonic Azoxymethane-induced Tumors: Effects on Mitogen-activated Protein Kinase, Cyclooxygenase-2, and Cyclin D1

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ABSTRACT

Azoxymethane (AOM)-induced colonic carcinogenesis involves a number of mutations, including those in the K-ras gene and CTNNB1, that codes for β-catenin. Prior in vitro studies have also demonstrated that wild type p21K-ras can be activated by epigenetic events. We identified 15 K-ras mutations in 14 of 84 AOM-induced colonic tumors by three independent methods. By single strand conformational polymorphism, we also observed mutations in 22 of 68 tumors in exon 3 of CTNNB1. A highly sensitive method was then used to measure p21ras activation levels. All tumors assayed possessing K-ras mutations had significantly higher p21ras activation levels (8.8 ± 1.5%; n = 13) compared with that of control colon (3.7 ± 0.4%; n = 6; P < 0.05) or tumors without such mutations (4.2 ± 0.4%; n = 70; P < 0.05). Among tumors with wild-type K-ras, there was a subset of tumors (18 of 70 that had significantly higher p21ras activation levels (8.0 ± 0.9%; n = 18) compared with control colon. In three of four tumors examined with activated wild-type p21ras, we observed increased c-erbB-2 receptor expression and decreased Ras-GAP expression. In contrast, only one of eight tumors examined with wild-type ras and nonactivated p21ras demonstrated these alterations. Mitogen-activated protein kinase (MAPK) activation and cyclooxygenase-2 (COX-2) expression were increased in tumors with mutated or activated wild-type p21ras, compared with their nonactivated counterparts. Although β-catenin mutations did not alter COX-2 expression or MAPK activity, mutations in either K-ras or β-catenin significantly increased cyclin D1 expression. In contrast, in tumors with wild-type but activated p21ras, cyclin D1 expression was not enhanced. Thus, the spectrum of changes in MAPK, COX-2, and cyclin D1 is distinct among tumors with ras or β-catenin mutations or nonmutational activation of p21ras.

INTRODUCTION

Colonic malignant transformation involves activating mutations in proto-oncogenes, such as K-ras, and genetic alterations in tumor suppressor genes, including the APC gene and CTNNB1, the gene coding for β-catenin, a downstream effector of APC signaling (1–4). K-ras mutations have been detected in ~50% of large human colonic adenomas and adenocarcinomas (2), and mutations in APC or β-catenin occur in most colon cancers (1). Mutations in the K-ras gene (5, 6) and in CTNNB1 (7) can also be detected in colonic tumors of rats administered the colonic procarcinogen, 1,2-dimethylhydrazine, or its proximate metabolite, AOM.

Several lines of evidence from both human (8–10) and experimental systems (11–14) have also indicated that epigenetic alterations in a number of important signal transduction elements, including cyclin D1 and p21ras, may lead to the clonal expansion of a variety of malignant cell types. Recent in vitro studies, for example, have found that expression of cyclin D1 is increased by mutations in K-ras or APC/β-catenin (15–17). Cyclin D1 activates cyclin-dependent kinase-4 and cyclin-dependent kinase-6 and, thereby, promotes the G1 to S transition. This growth enhancing regulator is also increased in a subset of AOM-induced tumors (18).

The K-ras proto-oncogene codes for p21K-ras, a small monomeric GDP/GTP-binding (G) protein, which is involved in the regulation of a number of important normal cellular functions, including proliferation, differentiation, and apoptosis (19, 20). In its GDP-bound form, it serves as an active signal transducer, whereas in its GDP-bound state, p21K-ras is inactive (20, 21). The conversion of GTP-bound to GDP-bound p21K-ras is stimulated by GAPs (20). Inactive p21K-ras, in turn, can be reactivated by replacement of its bound GDP by GTP, via GNEFs (20, 21). Several receptor tyrosine kinases, including EGF and c-erbB-2 receptors, have been shown to activate p21ras by increasing GNEF activity (22, 23). Although GAPs and GNEFs can, thereby, regulate the activation of wild-type p21K-ras, these two regulatory proteins do not influence oncogenic p21K-ras, which is constitutively activated by mutation (20). Because only ~50% of colonic carcinoma have mutant p21K-ras, we hypothesized that alterations in the activity and/or expression of Ras-GAP and/or GNEFs, such as Sos, could lead to sustained activation of wild-type p21K-ras in colonic malignant cells not possessing K-ras mutations. To date, however, these possibilities have not been experimentally demonstrated in models of colonic carcinogenesis in vivo.

Activation of p21K-ras, in turn, might be expected to stimulate a number of its downstream effectors including, for example, the ERK family of MAPK by activating the MEK kinase, Raf-1, thereby phosphorylating and activating MEK (reviewed in Ref. 24). This latter dual functioning kinase activates two isoforms of MAPK/ERK, pp44ERK1 and pp42ERK2. Once activated, these MAPKs translocate to the nuclei of cells, and their phosphorylated substrates, in turn, lead to transactivation of genes involved in the regulation of cellular proliferation, differentiation, and malignant transformation (25). We, therefore, determined whether the activation status of p21K-ras in AOM-induced tumors was related to alterations in the activities of these MAPKs.

The gene coding for COX-2, an inducible isof orm of the enzyme that catalyzes the conversion of arachidonic acid to prostaglandins and other eicosanoids (26), is another important downstream target of p21K-ras. COX-2, for example, has been shown to be increased in a number of ras-transformed epithelial cells (27). Several lines of evidence, moreover, have implicated COX-2, which has been shown
to be overexpressed in both human (28) and experimental colonic tumors (29, 30), in colonic malignant transformation (31, 32). Although no direct evidence has emerged linking aberrant APC/β-catenin signaling to alterations in COX-2, recent in vitro studies in Rat-1 fibroblasts transfected with an activated Ha-ras oncogene have shown that increases in MAPK activity appear to be required for the induction of COX-2 expression by this proto-oncogene (27). We, therefore, examined the possible relationship between the activity of p21\textsuperscript{ras} and the expression of COX-2 and the activities of MAPKs in AOM-induced tumors. In addition, this model allowed us to investigate whether MAPK activity and COX-2 and cyclin D1 expression would be altered by β-catenin mutations.

### MATERIALS AND METHODS

**Materials.** Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) provided monoclonal anti-Ras GAP (B4F8), anti-pan p21\textsuperscript{ras}, and rabbit polyclonal antibodies to Sos1 and Sos2 (D21), β-catenin, and to EGFR. Rabbit polyclonal anti-active MAPK antibodies (pTyr202/pTyr204) were from Promega Corp. (Madison, WI). Anti-COX-2 polyclonal antibodies were from Cayman Chemical (Ann Arbor, MI). Rabbit polyclonal anti-cyclin D1 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit antihuman c-erbB-2 antibodies and the alkaline phosphatase-labeled LSAB kit were from DAKO (Carpentaria, CA). Caco-2 cells, transfected with COX-2, were generously provided by Dr. R. Dubois (Vanderbilt University). Unless noted otherwise, all other reagents were of the highest quality available and were obtained from Sigma.

**Experimental Protocol.** Weanling male albino Fisher (F344) rats, initially weighing 80–100 g, were fed a standard rat chow diet (#5001, Purina Mills, Richmond, IN). Two weeks after being placed on this diet, one-half of the rats received i.p. injections weekly with AOM (15 mg/kg body weight/week) or vehicle (saline) for 2 weeks and then maintained on the diet for an additional 35 weeks. At that time, the animals were sacrificed in the nonfasted state. Colons were removed, flushed with normal saline, opened, and examined macroscopically for the presence of tumors. Tumors were rapidly excised and washed with ice-cold PBS. A small portion from each tumor was fixed in 10% buffered formalin for microscopic examination, whereas the remainder was snap frozen in liquid nitrogen for later analyses. After formalin fixation, tissue specimens were paraffin embedded, sectioned, and stained with H&E, as described previously (33). All specimens were evaluated by a pathologist (J. H.), who was unaware of the treatment groups. Macroscopic lesions were classified as either benign (adenoma) or malignant (adenocarcinoma). No tumors were present in the group treated with vehicle. Tissue specimens of colon mucosa, −1 cm\textsuperscript{2} in size, were taken from each of these control rats and processed in the same way as tumor samples.

**DNA Isolation, PCR Amplification, and Allele-specific Oligonucleotide Hybridization.** The snap-frozen samples were mechanically disrupted, and DNA was extracted using the TRI Reagent. In these samples, a 116-bp sequence of the K-ras exon 1 gene was amplified by PCR and then ASOH was carried out as described previously (6). To investigate G→A mutations in the second position of codon 59 or in the first position of codons 12 or 13, appropriate primers and conditions were used as described (6).

**Standard and Enriched Primer-mediated RFLP.** To confirm the ASOH results, PM-RFLP was used to detect K-ras mutations in codons 12 and 13. Briefly, mismatched 5′ primers created restriction sites for BstN1, or BglII in wild-type K-ras, which were abolished by G→A mutations in codon 12 or 13, respectively. The primers, PCR conditions, and restriction digestions were as described previously (5). To amplify low abundance codon 12 K-ras mutant forms, a procedure of enriched PM-RFLP was carried out as described previously (34).

**PCR-SSCP Analysis.** Primers for PCR were designed to amplify the consensus sequence for GSK-3β phosphorylation in exon 3 of CTNNB1, the gene coding for β-catenin, based on the published cDNA sequence of mouse and used successfully in rat (7). PCR and subsequent SSCP was performed exactly as described previously (7).

**DNA Sequencing.** K-ras and β-catenin mutations were also confirmed by direct sequencing of amplified DNA using automated fluorescent DNA sequencing.

**Results.**

**K-ras Mutations in AOM-Induced Colonic Tumors.** To assess tumors for K-ras mutations, we initially used an ASOH technique, as described previously by our laboratory (6). Fifteen K-ras mutations were observed in 14 of 84 AOM-induced colonic tumors (16.7%). Of the K-ras mutations, 9 occurred in codon 12 and 4 in codon 13, and one tumor had mutations in both codons 12 and 13. No mutations, however, were present in codon 59. These mutations were all confirmed by PM-RFLP (5), as well as by direct sequencing of the PCR products (data not shown). There was also complete agreement between ASOH and PM-RFLP techniques in identifying tumors expressing wild-type K-ras.

**CTNNB1 Mutations in AOM-Induced Colonic Tumors.** Among the 84 tumors screened for K-ras mutations, 68 tumors were also examined for CTNNB1 mutations by SSCP. We identified 22 mutations (32.4%) in exon 3 of this gene coding for β-catenin (Fig. 1 and Table 1). There were no significant correlations between K-ras and CTNNB1 mutations (Table 1). In agreement with others (7), we also found an increased expression of β-catenin and a redistribution of the protein from the membrane to the cytosol and nucleus in tumors with mutant β-catenin (Fig. 1).

**Activation State of p21\textsuperscript{ras} in AOM-Induced Tumors with or without K-ras Mutations.** Activating K-ras mutations code for mutant p21\textsuperscript{K-ras}, which is characterized by increased GTP binding. In addition to activating K-ras mutations, other nonmutational mechanisms have been reported to cause increased p21K-ras activity (9, 12, 13, 39). We, therefore, asked whether nonmutational activation of p21K-ras occurred in AOM-induced tumors expressing wild-type K-ras. We measured p21\textsuperscript{ras} activity, as assayed by the percentage of GTP to total (GTP + GDP) bound to p21\textsuperscript{ras}. The p21\textsuperscript{ras} activation ratios of the colonic tumors assayed with mutant K-ras (8.8 ± 1.5; n = 13) were significantly higher than that of the nonmutant group (4.2 ± 0.4; n = 70; P < 0.05; Table 2). Interestingly, as shown in...
ACTIVATION OF WILD-TYPE p21<sup>ras</sup> IN AOM TUMORS

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Table 1  Summary of K-ras and CTNNB1 mutations in AOM-induced colonic tumors

<table>
<thead>
<tr>
<th>K-ras</th>
<th>CTNNB1</th>
<th>Tumors (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>41 (60.3%)</td>
</tr>
<tr>
<td>Mutant</td>
<td>Wild type</td>
<td>5 (7.4%)</td>
</tr>
<tr>
<td>Wild type</td>
<td>Mutant</td>
<td>16 (23.5%)</td>
</tr>
<tr>
<td>Mutant</td>
<td>Mutant</td>
<td>6 (8.8%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> % of total tumors (n = 68) assayed for both K-ras and CTNNB1 mutations.

Table 2  Summary of p21<sup>ras</sup> activation in control colonocytes and AOM-induced colonic tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>% Ras GTP:[GDP + GTP]</th>
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<tr>
<td>Control colonocytes</td>
<td>6</td>
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<tr>
<td>Tumors with wild-type K-ras</td>
<td>70</td>
<td>4.2 ± 0.4</td>
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<tr>
<td>Tumors with K-ras mutations</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.8 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tumors with constitutive activation of wild-type p21&lt;sup&gt;ras&lt;/sup&gt;</td>
<td>18</td>
<td>8.0 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Only 13 of the 14 tumors with K-ras mutations were available for p21<sup>ras</sup> activation measurements.

<sup>b</sup> P < 0.05, compared with control colonocytes or tumors with wild-type K-ras.

To address possible mechanisms involved in the activation of wild-type p21<sup>ras</sup> in AOM-induced tumors without K-ras mutations, we examined the protein expression of GAP and the GNEF, Sos-1, in colonic adenocarcinomas with activated p21<sup>ras</sup> and compared their expression to that present in adenocarcinomas with wild-type p21<sup>ras</sup> and normal levels of p21<sup>ras</sup> activation (Fig. 2). There was an inverse correlation between GAP expression and p21<sup>ras</sup> activation, with decreased GAP expression in tumors with wild-type but activated p21<sup>ras</sup> (Fig. 2B). In contrast, GAP expression was elevated in tumors with mutant (GAP-unresponsive) p21<sup>K-ras</sup> compared with tumors with nonactivated wild-type p21<sup>K-ras</sup> (data not shown). These findings suggest that a decrease in the expression of GAP may, at least in part, underlie the activation of wild-type p21<sup>ras</sup> in a subset of AOM-induced tumors without K-ras mutations, whereas the GAP elevation in the K-ras mutant tumors may possibly reflect a futile attempt to inhibit their activated ras state. The expression of Sos-1, however, was not different in tumors with (134 ± 13%; n = 4) and without (100 ± 17%; n = 4; P > 0.10) constitutively activated p21<sup>ras</sup> (Fig. 2C). Sos-2 was not detected in normal or neoplastic tissue (Fig. 2C).

We next examined tumors in each of the two aforementioned groups, as well as tumors with K-ras mutations, with respect to their expression of EGF and c-erbB-2 receptors. Studies in several cell types have demonstrated that stimulation of these tyrosine kinase receptors can lead to p21<sup>ras</sup> activation (40–43).

Table 2  Summary of p21<sup>ras</sup> activation in control colonocytes and AOM-induced colonic tumors

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<sup>b</sup> P < 0.05, compared with control colonocytes or tumors with wild-type K-ras.
carninomas with wild-type but activated p21\textsuperscript{ras} had detectable expression of the c-erbB-2 receptor, compared with only one of eight adenocarcinomas with normal levels of p21\textsuperscript{ras} activation (Fig. 3). These three tumors with increased c-erbB-2 receptor expression also had low GAP expression (see above). Moreover, the remaining single tumor, in the group with nonmutant \textit{ras} and activated p21\textsuperscript{ras}, also had decreased GAP expression. In contrast, four of four tumors examined with mutant \textit{K-ras} were negative for c-erbB-2 staining (Fig. 3). EGF receptor expression was not different among the three groups (data not shown). These findings suggest that c-erbB-2 receptor overexpression may also account, at least in part, for the increased GTP binding to wild-type p21\textsuperscript{ras} in the subset of AOM-induced tumors without K-
\textit{ras} mutations. Because tumors with mutant \textit{K-ras} did not show increased c-erbB-2 receptor expression (Fig. 3), this suggests that increased receptor expression drives increased p21\textsuperscript{ras} activation and not the converse.

The Activation State of p21\textsuperscript{ras} Influences the Activities of MAPKs and the Expression of COX-2. AOM-induced tumors with either mutated p21\textsuperscript{ras} or activated wild-type p21\textsuperscript{ras} also had significantly greater activation of ERK-1 and ERK-2, compared with those with normal (nonactivated) wild-type p21\textsuperscript{ras}, as assessed by Western blotting with antibodies specific for the phosphorylated (activated) forms of ERK1/2 (Fig. 4). In tumors with wild-type but activated p21\textsuperscript{ras}, ERK-1 and ERK-2 were 222 \pm 26\% and 104 \pm 18\% above the levels of ERK-1 and ERK-2 from tumors with normal p21\textsuperscript{ras} activation (n = 4; P < 0.05), respectively. Tumors with mutant p21\textsuperscript{ras} also had increased ERK-1 and ERK-2 activation, 156 \pm 15\% and 223 \pm 8\% above that of ERK-1 and ERK-2 from tumors with wild-type nonactivated p21\textsuperscript{ras} (n = 4; P < 0.05), respectively. There were no differences in the total expression of ERK-1 or ERK-2 among the three groups (data not shown). As shown in Fig. 4B, there were significant correlations between phospho-ERK-1 and phospho-ERK-2 and p21\textsuperscript{ras} activation.

In agreement with earlier studies in the AOM model (30), we found an increase in the expression of COX-2 in all tumors compared with normal colonic mucosa (Fig. 5). There were no significant differences in COX-2 expression between tumors with mutant \textit{versus} wild-type \textit{\beta-catenin} (Fig. 5B). In contrast, the expression of COX-2 was significantly greater in tumors with activated p21\textsuperscript{ras} (mutated or wild-type) compared with tumors with wild-type but nonactivated p21\textsuperscript{ras} (Fig. 5A). Interestingly, in tumors with wild-type but constitutively activated p21\textsuperscript{ras}, the expression of COX-2 was diffuse and predominantly cytosolic (Fig. 6, E–H), as well as present in the surrounding stroma, whereas in tumors with mutant \textit{K-ras}, there was increased nuclear COX-2 expression in the malignant epithelium (Fig. 6, I–L). The mechanism for this difference in distribution is currently unclear and will require further study.

Mutations in \textit{\beta-catenin or ras} but not Activation of Wild-Type p21\textsuperscript{ras} Increase Cyclin D1. In contrast to the differential effects of mutations in \textit{\beta-catenin} compared with \textit{K-ras} on MAPK and COX-2, both of these mutations increased cyclin D1 expression, as demonstrated by Western blotting (Fig. 7) and immunohistochemical analysis (Fig. 8). Tumors with wild-type \textit{K-ras} but activated p21\textsuperscript{ras}, however, did not show increased cyclin D1 expression (Figs. 7 and 8). Thus, activation of p21\textsuperscript{ras}, by mutational \textit{versus} nonmutational mechanisms, causes parallel changes in MAPK activation but differential alterations in cyclin D1 expression and COX-2 localization. Our findings suggest that the signaling cascades involved in activation of wild-type p21\textsuperscript{ras} may block the expected increase in cyclin D1 by MAPK that we observed in tumors with mutant \textit{K-ras}. Elucidation of the underlying mechanisms for these differences in cyclin D1 expression in tumors with
mutant versus nonmutant activated p21<sub>ras</sub> will, however, require further study.

**DISCUSSION**

These studies demonstrate for the first time that a subset of AOM-induced rat colonic tumors (18 of 84; 21.4%), without detectable K-ras mutations, have constitutively activated wild-type p21<sub>ras</sub>. Moreover, wild-type p21<sub>ras</sub> was found to be activated in 20% (7 of 35) of AOM-induced adenocarcinomas, compared with 45.4% (10 of 22) adenomas and 7.6% (1 of 13) of tumors with carcinoma in situ. The frequencies of wild-type activated p21<sub>ras</sub> and mutant p21<sub>ras</sub> are comparable, occurring in 21.4 and 16.7% of AOM-induced tumors, respectively, in this model of colonic carcinogenesis. The frequency of K-ras mutations in this study is similar to that of Vivona et al. (44) but lower than that in other studies (5, 45) using comparable AOM treatment regiments. The explanation for these differences is not apparent. Further studies will, therefore, be necessary to address the relative contributions of activated wild-type versus mutant p21<sub>ras</sub> in the development of colonic carcinomas.

The ratio of GTP:(GTP + GDP) bound to p21<sub>ras</sub> in the group with wild-type ras, but with activated p21<sub>ras</sub>, and the group with mutated p21<sub>ras</sub>, were similar, suggesting that like mutated p21<sub>ras</sub>, activated wild-type p21<sub>ras</sub> may also be intimately involved in colonic malignant transformation by altering critical cellular processes (46, 47). Because wild-type p21<sub>ras</sub> was constitutively activated in both adenomas and adenocarcinomas, the constitutive activation of p21<sub>ras</sub> appears to occur early in colonic malignant transformation. Prior studies have shown that wild-type p21<sub>ras</sub> was activated in human peripheral nerve sarcomas, although the ras mutational status of these tumors was not assessed (10). In addition, in several ovarian carcinoma cell lines without mutant ras, wild-type p21<sub>ras</sub> was found to be activated (39). The present studies are the first, however, to demonstrate the activation of wild-type p21<sub>ras</sub> in any animal model of carcinogenesis.

As noted earlier, the activation state of wild-type p21<sub>ras</sub> is normally regulated by changes in the activity and/or expression of GAP and GNEFs, such as Sos. A decrease in the activity/expression of GAP or an increase in the activity/expression of GNEFs would be expected to activate wild-type p21<sub>ras</sub>. Indeed, we found that GAP expression was inversely proportional to levels of wild-type p21<sub>ras</sub> activation, whereas Sos-1 expression was not significantly different between tumors with activated and nonactivated p21<sub>ras</sub> activation. Because of the limited quantities of these tumors, the enzymatic activities of GAP and Sos-1 could not be assessed and will be of interest to examine in future experiments. Although the underlying mechanism(s) responsible for the decrease in GAP expression remains to be elucidated, it would appear that alterations in GAP expression, at least in part, are responsible for the activation of wild-type p21<sub>ras</sub> in this subset of adenocarcinomas.

Alterations in growth factors and/or their receptors, which regulate GAP and GNEF activity, may be involved in activation of wild-type p21<sub>ras</sub>. For example, tumor-associated increases in growth factors could stimulate their respective tyrosine kinase receptors and, thereby, activate p21<sub>ras</sub> (41, 42). Alternatively, overexpression of these receptors could also lead to p21<sub>ras</sub> activation (40). Recent studies in human colon cancer and in several colonic cancer-derived cell lines have demonstrated increased c-erbB-2 receptor expression (48, 49). In the
Fig. 6. COX-2 expression by IHC in AOM-induced tumors. Sections were immunostained for COX-2 expression as described in “Materials and Methods." For each tumor, the p21\textsuperscript{ras} activation ratio is given. Tumors A–D were wild type for \( \beta \)-catenin, and K-ras and had normal p21\textsuperscript{ras} activation ratios: A, 2.3; B, 2.1; C, 2.1; D, 1.9. Tumors E–H were wild type for \( \beta \)-catenin and K-ras but had elevated p21\textsuperscript{ras} activation ratios: E, 8.4; F, 9.2; G, 5.9; H, 5.1. Tumors I–L were wild type for \( \beta \)-catenin and mutant for K-ras: I, 6.2; J, 17.9; K, 13.5; L, 5.5. Note increased cytoplasmic staining for COX-2 in the tumors with nonmutational activation of p21\textsuperscript{ras} (e.g., arrowhead, E), as well as increased staining in the stromal cells of these tumors (e.g., open arrowhead, G). In contrast, tumors with mutant K-ras have increased nuclear staining for COX-2 (e.g., closed arrows, J).

Present studies, c-erbB-2 receptors were detectable in three of four AOM-induced tumors with activated p21\textsuperscript{ras} compared with 1 of 8 with nonactivated p21\textsuperscript{ras}. Because the overexpression of this receptor has been shown to activate p21\textsuperscript{ras} (40), alterations in the expression of c-erbB-2 receptors may, at least in part, along with decreases in the expression of GAP, be responsible for the activation of wild-type p21\textsuperscript{ras}.

The present studies also demonstrate that colonic tumors with activated wild-type p21\textsuperscript{ras}, like those with mutated p21\textsuperscript{ras}, have increased activation of ERK-1 and ERK-2, presumably via the activation of Raf-1 and MAPK kinase (46, 47). Thus, activated wild-type p21\textsuperscript{ras}, like their mutated counterparts, may be involved in colonic malignant transformation, because activation of these MAPKs may be expected to influence the proliferation and differentiation of these malignant cells (46, 47). Recent studies, moreover, have demonstrated that blockade of MAPK activation can inhibit colonic tumor growth in vivo (50).

In agreement with earlier studies in the AOM model (30), we found that COX-2 was increased in all of these carcinogen-induced tumors. In agreement with prior in vitro studies (27), our results also suggest that K-ras mutations, via increases in the activities of MAPKs, may lead to COX-2 overexpression in tumors. Furthermore, our studies indicate that this also occurs in tumors with activated wild-type p21\textsuperscript{ras}. The cytoplasmic localization of COX-2 in the stromal and malignant cells of tumors with wild-type activated p21\textsuperscript{ras}, compared with a nuclear distribution in malignant cells with mutant K-ras, however, underscores important differences in COX-2 expression depending upon the mechanism of p21\textsuperscript{ras} activation. Further studies will be required to understand these differences that may arise from signal transduction pathways driving p21\textsuperscript{ras} activation in the case of wild-type ras, compared with mutant p21\textsuperscript{ras}.

In addition to alterations in ras and its effectors, changes in APC and \( \beta \)-catenin signaling have been identified in colonic carcinogenesis. A number of downstream targets of APC/\( \beta \)-catenin have been demonstrated in human colonic adenocarcinoma cells, including cyclin D1 (51, 52). We have found that both K-ras and CTNNB1 mutations can drive increased cyclin D1 expression in vivo, in agreement with previous in vitro findings in cultured cell lines (15–17). The failure of cyclin D1 to increase in tumors with wild-type but activated p21\textsuperscript{ras} suggests that the signaling involved in this activation may also inhibit the expected increased cyclin D1 expression induced by the downstream ras effector pathway Raf-MEK-MAPK. The mechanisms involved in this inhibition will also require further study.

Although COX-2 expression is increased in most human sporadic colon cancers, we did not find that \( \beta \)-catenin mutations altered COX-2 expression in the AOM model. This is in agreement with recent in vitro studies with colon cancer-derived HT29 cells in which peroxisome proliferator-activated receptor \( \gamma \) agonists of the thiazolidinedione class increased \( \beta \)-catenin expression but failed to alter COX-2 expression (53). Independent of the \( \beta \)-catenin status, however, it should be noted that COX-2 expression was increased in all AOM-induced tumors examined, compared with normal mucosa.

In summary, the present results demonstrate that a subset of AOM-induced tumors possess activated wild-type p21\textsuperscript{ras}, perhaps as a consequence of changes in the expression of c-erbB-2 receptors and/or GAP. Moreover, like their activated, mutated p21\textsuperscript{ras} counterparts, these activated wild-type small monomeric G proteins appear to play a role in colonic malignant transformation via increases in the activities of the MAPKs which, in turn, may lead to an increase in

Fig. 7. Cyclin D1 expression by Western blotting in AOM-induced tumors. Tumor lysates were prepared, and proteins were probed by Western blotting for cyclin D1 expression as described in “Materials and Methods.” A, effects of K-ras mutational status and p21\textsuperscript{ras} activation on cyclin D1 expression. B, effect of \( \beta \)-catenin mutational status on cyclin D1 expression.

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COX-2 expression. Compared with mutational activation of p21\textsuperscript{ras}, the differential alterations in cyclin D1 expression and COX-2 localization observed in tumors with nonmutational activation of p21\textsuperscript{ras} may involve alternative signaling pathways that activate the gene product of wild-type but not mutant ras. Elucidation of the mechanisms that drive these differences may identify novel new targets for anticancer therapy.

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Mutational and Nonmutational Activation of p21ras in Rat Colonic Azoxy methane-induced Tumors: Effects on Mitogen-activated Protein Kinase, Cyclooxygenase-2, and Cyclin D1

Marc Bissonnette, Sharad Khare, Friederike C. von Lintig, et al.


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