APC and Oncogenic *KRAS* Are Synergistic in Enhancing Wnt Signaling in Intestinal Tumor Formation and Progression

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Background & Aims: Synchronous activation of the Wnt signaling pathway, mostly because of loss of function of the *APC* tumor suppressor, and of the oncogenic *KRAS*-signaling pathway is very frequent in colorectal cancer and is associated with poor prognosis. **Methods:** We have generated a compound transgenic mouse model, $KRAS^{V12G}/Apc^{+/1638N}$, to recapitulate the human disease and compared it with single transgenic littermates. **Results:** Compound mutant mice are characterized by a 10-fold increase in tumor multiplicity and by accelerated tumor progression, resulting in strongly enhanced morbidity and mortality. Tumors from compound mutant mice proliferate faster and show decreased levels of apoptosis. Several lines of evidence indicate that the observed increase in tumor multiplicity and malignant transformation is caused by the synergistic activation of Wnt signaling in cells with oncogenic *KRAS* and loss-of-function *Apc* mutations. Activated *KRAS* is known to induce tyrosine phosphorylation of β-catenin, leading to its release from E-cadherin at the adherens junction. This results in an increased β-catenin pool in the cytoplasma, its subsequent translocation to the nucleus, and the transcriptional activation of Wnt downstream target genes. Accordingly, intestinal tumors from $KRAS^{V12G}/Apc^{+/1638N}$ mice show a significant increase in cells with nuclear accumulation of β-catenin when compared with $Apc^{+/1638N}$ animals. Moreover, *Apc*/*KRAS*-mutant embryonic stem cells show a significantly enhanced β-catenin/T-cell factor–mediated transcriptional activation, accompanied by increased β-catenin nuclear localization. **Conclusions:** This *KRAS*-induced increase in Wnt/β-catenin signaling may enhance the plasticity and self-renewal capacity of the tumor, thus resulting in the drastically augmented tumor multiplicity and malignant behavior in compound mutant animals.

Colorectal cancer (CRC) and in particular the adenoma-carcinoma sequence still represents a paradigm for the molecular and genetic mechanisms underlying tumor formation and progression.[1,2] Cancers of the colon and rectum are among the most frequent cause of morbidity and mortality among Western industrialized countries.[3,4] In the vast majority of sporadic CRC cases, mutations in genes known to play rate-limiting roles in the canonical Wnt/β-catenin signal transduction pathway such as the adenomatous polyposis coli (*APC*) tumor suppressor gene and the β-catenin (*CTNNB1*) oncogene trigger adenomatous polyp formation, the first step toward colorectal neoplasia.[5] Activating mutations of the *KRAS* oncogene accompany adenoma growth and progression, whereas loss of heterozygosity (LOH) and mutations at the *SMAD4* and *TP53* tumor suppressor genes underlie malignant transformation at later stages.[1] Loss-of-function mutations at *APC* have been observed in more than 60% of colonic adenomas and carcinomas.[6] Also, germ-line *APC* mutations are responsible for familial adenomatous polyposis (FAP), an autosomal dominant predisposition to the development of multiple colorectal polyps.[7] Loss of APC function results in constitutive activation of Wnt signaling because of impaired β-catenin down-regulation, leading to its cytoplasmic accumulation and nuclear translocation.[8,9] In the nucleus, upon association with members of the T-cell factor (TCF) family of transcriptional activators, β-catenin differentially modulates the expression of Wnt downstream target genes implicated in cell proliferation, migration, differentiation, and apoptosis[10] (http://www.stanford.edu/~rnusse/pathways/ targets.html[11]). Approximately 50% of colorectal adenomas and carcinomas carry activating mutations of the *RAS* protooncogene.[1,12] The *KRAS* gene is an effective marker for molecular diagnosis and tumor progression in colorectal, pancreas, and lung cancer. Oncogenic RAS proteins are locked in their guanosine triphosphate (GTP)-bound (active) form and mediate their tumorigenic effects through multiple downstream effectors, the most prominent of which, the RAS effector RAF kinase, activates on its turn the extracellular signal-regulated kinase (ERK)-mitogenactivated protein (MAP) kinase (MAPK) cascade.[13–15] Activated MAP kinases phosphorylate downstream transcription factors, thus inducing the expression of regulatory genes required for entry into the S phase of the cell cycle.[16,17] Thus, activation of the Wnt as well as of the RAS signal transduction pathways plays a rate-limiting role in human CRC formation and progression. Both *APC* and *KRAS* mutations occur in aberrant crypt foci, microscopic precursor lesions that have been postulated to precede the development of adenomatous polyps.[18] APC mutations are associated with dysplasia in small precursor lesions, whereas KRAS mutations are more often found in nondysplastic lesions.[19] Also, synchronous detection of activated KRAS and of β-catenin nuclear accumulation, the hallmark of canonical Wnt-signaling activation, identifies a group of CRC patients with poor prognosis and

Abbreviations used in this paper: APC, adenomatous polyposis coli; CRC, colorectal cancer; CSC, cancer stem cell; ES, embryonic stem; GTP, guanosine triphosphate; LOH, loss of heterozygosity; MAPK, mitogen-activated protein kinase; TCF, T-cell factor; WT, wild-type.

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resistance to standard chemotherapy.[20] However, whether the frequent concurrent activation of *RAS* and the Wnt pathways in colorectal tumors is due to a cumulative effect or to a more synergistic interaction between the 2 signaling cascades is still largely unknown. To address this clinically relevant issue, we have generated a mouse model that carries both a targeted loss-of-function mutation at the endogenous *Apc* gene[21], Apc^{1638N}, and a transgene encoding for the activated form of the human *KRAS* oncogene, the pVillin-$KRAS^{V12G}$ (hereafter further referred to as $KRAS^{V12G}$).[22] Compound animals are characterized by a striking increase in intestinal tumor multiplicity and progression, leading to high morbidity and mortality. Molecular analysis of these tumors and of primary cell lines carrying both mutations indicate that enhanced Wnt-signalling activity by oncogenic KRAS is likely to underlie the increased tumor initiation and progression toward malignancy in the compound *Apc*/*KRAS* animals.

Materials and Methods

Animal Models

All experiments on mice were performed in accordance with institutional and national guidelines and regulations. The Apc^{1638N} mouse lineage in the inbred C57Bl/6J background[21] was bred with the transgenic model[22] pVillin-$KRAS^{V12G}$ in the genetic background B6D2 (C57Bl/6J × DBA/2). To control for genetic background effects, littermates were always used as controls. Mice were maintained under a 12-hour light-dark cycle and fed with standard diet and water ad lib. Genotyping was performed on DNA extracted from mouse tails as previously described.[22,23]

Tumor Analysis and Tissue Processing

The median age of the analyzed animals was 5.5 months ($KRAS^{V12G}/Apc^{+/1638N}$), 7 months ($Apc^{+/1638N}$), and 9 months ($KRAS^{V12G}$). Animals were killed at the ages indicated or at the appearance of signs of distress, and the gross study of the tissues was carried out as described.[22] Macroscopically visible tumors were resected and embedded in paraffin according to standard procedures. Tumors were classified according to standard World Health Organization (WHO) histopathologic criteria by an experienced pathologist. In addition to the processing for histopathologic analysis, a subset of freshly isolated tumors was also snap frozen in liquid nitrogen and stored at −80°C. Frozen tumors were either used for DNA/RNA extraction (Qiagen, Hilden, Germany) or embedded in Tissue-Tek (Sakura B.V., Zoeterwoude, The Netherlands) and processed for cryosections. Kidneys, liver, and lungs of all animals were also investigated for the presence of metastases by macroscopic and, in a selected number of cases, microscopic analysis of serial sections. For protein analysis, snap-frozen mouse tissue or scrapings of intestinal mucosa were lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L benzamidine, 1 mmol/L PMSF, 1 mmol/L DTT, 2 mmol/L EGTA, 1% Triton X-100, 1% NP-40, Mammalian Protease Inhibitor Cocktail; Sigma Chemical Co, St. Louis, MO) using a 1-mL Dounce Homogenizer. After centrifugation (15,000*g*, 15 minutes, 4°C), supernatants were collected, and protein concentration was determined (Bio-Rad assay, Richmond, CA).

LOH Analysis

LOH of the *Apc* and *Tp53* genes was determined by PCR amplification of dinucleotide repeat markers.[22,24] DNA was isolated from microdissected tumors and from normal intestinal tissue with an RNA/DNA extraction kit (Qiagen). Primer sequences were obtained from the Mouse Genome Database.[22,25] The following polymorphic markers were used for the *Apc* locus: D18Mit64, D18Mit111, D18Mit132, and D18Mit17 and for the *Tp53* locus: D11Mit4, D11Mit30, and D11Mit278. LOH was defined at $P < .05$ for 3 independent PCR reactions.[22,26] A functional assay previously described was used to validate the pathogenicity of molecular changes at the Tp53 gene.[22,26,27]

RT-PCR Analysis of Wnt Downstream Targets in Intestinal Tumors

C-myc and cyclin D1 messenger RNA (mRNA) expression levels were analyzed in normal mucosa and tumors (n = 5 mice/genotype). RNA was harvested from snap-frozen tissues using the RNeasy extraction kit (Qiagen). Up to 2 μg of total RNA was then subjected to reverse transcription using Superscript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA) and oligo dT primers (pd(N)6, Roche, Mannheim, Germany). Reactions were carried out in SybrGreen PCR Master mix (Applied Biosystems, Courtaboeuf Cedex, France) under recommended conditions, run on ABI PRISM 7900, and analyzed with Sequence Detector Software (Applied Biosystems). Relative quantities were calculated using the ddCT formula and normalized to the transcript levels of the housekeeping gene TATA binding protein (TBP). Assays were performed in triplicate. Primer sequences used were as follows: TBP: forward, CCACGGACAACTGCGTTGAT; reverse, GGCTCATAGCTACTGAACTG. c-myc: forward, TAGTGCTGCATGAGGAGACA; reverse, GGTTTGCCTCTTCTCCACAG. cyclinD1: forward, CACAACGCACTTTCTTTCCAG; reverse, CGCAGGCTTGACTCCAGAAG.

Detection of Liver Micrometastases by RT-PCR

Mouse livers were dissected under sterile conditions to avoid contamination. Liver RNA was extracted from different tissues using the RNA Now Kit (Ozyme, St Quentin, France). RT-PCR reactions were performed as previously described.[22,28] Primers were used that amplify specifically the transgenic $KRAS^{V12G}$ gene under control of the villin promoter. The sense primer is specific to the villin promoter, CAAGCCTGGCTCGACGGCC, and the antisense primer recognizes the coding sequence of the human $KRAS^{V12G}$ gene, ATTTGCGGCCGCTTTACATAATTACACACT, yielding a fragment of 400 base pair. PCR reactions were repeated twice for each sample, and RNA was extracted twice from each tissue to confirm the result. Direct sequencing of the fragment confirmed the identity of the transgenic $KRAS^{V12G}$.

Western Blot Analysis

Equal amounts (40 μg) of protein lysate were separated on 13% polyacrylamide gels and further subjected to immunoblotting according to standard procedures. Primary antibodies used were as follows: anti-pan-Ras (Transduction