[CANCER RESEARCH 58, 3101–3104, July 15, 1998]

Mutation of the Type II Transforming Growth Factor-β Receptor Is Coincident with the Transformation of Human Colon Adenomas to Malignant Carcinomas[1]

William M. Grady, Ashwani Rajput, Lois Myeroff, Dong F. Liu, KiHo Kwon, Joseph Willis, and Sanford Markowitz[2]

Departments of Internal Medicine [W. M. G., S. M.], Surgery [A. R.], and Pathology [D. F. L., J. W.], and Ireland Cancer Center [W. M. G., A. R., L. M., S. M.], Case Western Reserve University/University Hospitals of Cleveland; Department of Pathology [K. K.], Wade Park Veterans Affairs Medical Center; and Howard Hughes Medical Institute [S. M.], Cleveland, Ohio 44106

ABSTRACT

The transforming growth factor-β (TGF-β) type II receptor (*RII*) is a colon cancer suppressor gene that is inactivated by mutation in 90% of human colon cancers arising via the microsatellite instability (MSI) pathway of carcinogenesis. To determine the pathophysiological consequence of *RII* mutations, we have determined the timing of their onset among 22 MSI human colon adenomas of varying stages. No *RII* mutations were detected in any early MSI adenoma, including all those with simple tubular or villous histology. The earliest *RII* mutation detected was in a region of high-grade dysplasia but was absent from the surrounding simple adenoma. Six additional *RII* mutations were all found in highly progressed adenomas that contained regions of frankly invasive adenocarcinoma. These *RII* mutations were detected in both the advanced adenomas and their adjacent regions of carcinoma. *RII* mutation is a late event in MSI adenomas and correlates tightly with progression of these adenomas to cancer.

INTRODUCTION

TGF-β[3] is a potent negative regulator of epithelial cell growth, and in cell culture it can cause complete inhibition of the growth of nontransformed colon epithelial cells, as well as induction of apoptosis of these cells (1, 2). In contrast, full malignant transformation of colon epithelial cells is commonly associated with the acquisition of resistance to TGF-β (2–4). Indeed, previous studies by Manning and collaborators (4) and by our group (2) both showed that malignant progression of colon adenoma cell lines directly correlated with the acquisition of TGF-β resistance in these cultured cells. Molecular mediators of TGF-β resistance described in colon cancers include mutations in TGF-β *RII* (5, 6), as well as mutations in candidate TGF-β signal transduction molecules *Smad2* and *Smad4* (7–9). The first of these mutations to be described were *RII* frameshift mutations clustered within a microsatellite-like 10-bp polyadenine repeat within the *RII* coding region (the *BAT-RII* tract). Such *BAT-RII* frameshift mutations are characteristic of colon cancers that arise by the pathway of MSI, and biallelic inactivation of *RII* secondary to *BAT-RII* mutations can be found in 90% of MSI colon cancers (5, 6). MSI is the hallmark of colon cancers that arise in families with the familial cancer syndrome of HNPCC, in which individuals inherit defects in genes encoding components of the DNA MMR pathway. In these individuals, MSI and cancer arise in cells in which MMR activity is inactivated by loss of the residual wild-type MMR allele. More commonly, MSI has been found among sporadic colon neoplasms arising within the proximal (right) colon in individuals who, at least in some instances, have undergone somatic inactivation of both MMR alleles (10–13). Transfection of wild-type *RII* into an MSI colon cancer cell line bearing mutant endogenous *RII* alleles abrogated the ability of the cell line to form tumors in athymic mice (14). Thus, *RII* functionally acts as a tumor suppressor gene in these MSI colon cancers. Previous studies have not, however, ascertained the stage of colon carcinogenesis that *in vivo* is promoted by the MSI catalyzed *RII* mutations. To address this question, we have determined the timing of *BAT-RII* mutations relative to the multistep progression of benign colonic epithelium to neoplastic colonic adenoma and then to malignant colon carcinoma. We first assembled a collection of colon neoplasms with MSI that included representatives of each of the stages of colon neoplasia, including early adenomas with tubular or villous histology, progressed adenomas demonstrating regions of high grade dysplasia, and late adenomas that included regions of frankly invasive colon cancer. We then determined the presence of wild-type or mutant *BAT-RII* sequences in each of these neoplasms. We found that *RII* mutation is a feature of late adenomas and is tightly correlated with the transition of benign adenoma to malignant carcinoma.

MATERIALS AND METHODS

Tissue Microdissection and DNA Extraction. Seventy-one formalin-fixed, paraffin-embedded colonic adenomas were obtained by retrospective review of the pathology archives at University Hospitals of Cleveland or the Wade Park Veterans Affairs Medical Center. The adenomas were obtained from 57 different patients who had undergone either routine endoscopic polypectomy or a colonic resection for the treatment of an adjacent colonic adenocarcinoma. Microscopic assessment of adenomas was performed by J. W. or a staff pathologist at the Wade Park Veterans Affairs Medical Center. Adenomas were chosen principally from patients with neoplasms of the proximal (right) colon and were selected to achieve a roughly equal representation of different histological grades of adenoma. Adenomas showing regions of different histology (*e.g.*, regions of adenoma adjacent to high-grade dysplasia or to frankly invasive cancer) were microdissected to allow independent analysis of the distinct regions within the adenoma. The pathologist selecting adenomas, performing histological grading of adenomas, and microdissecting adenomas for extraction of DNA was blinded as to the results of the molecular studies.

DNA extraction was performed in a standard fashion by incubating the paraffin-extracted, rehydrated tissue in 50 mM Tris-HCl (pH 8.5) with 0.5% Tween 20 and 2.0 mg/ml proteinase K for 3 h at 55°C or by incubating the tissue in Instagene (Bio-Rad) and 3.0 mg/ml proteinase K for 3 h at 55°C. Extracted samples were aliquotted and stored at −20°C.

MSI. Many of the adenomas assessed in this study were removed by colonoscopic polypectomy, during which normal colonic mucosa was not obtained. For these adenomas, microsatellite shifts were examined using a PCR-based assay at the polyadenine mononucleotide repeats *BAT26* and *BAT40*, which we and others have previously shown are both relatively monomorphic in size distribution within the population and which are each >98% sensitive for the detection of MSI in colorectal cancers (6, 15). For these cases, the normal size distribution of *BAT26* and *BAT40* alleles was determined from a reference set of germ-line DNA from 10 normal individuals. Adenomas demonstrating a shift in either *BAT26* or *BAT40* were classified as demonstrating MSI. In cases for which matched normal tissue was available, the neoplasms were additionally typed at microsatellite loci *D2S123*, *D2S147*, and *D10S197*. However, for consistency, identification of MSI was, in all cases, based on the status of *BAT26* and *BAT40*.

The primers were 5′-TGACTACTTTTGACTTCAGCC-3′ and 5′-AAC-

Received 3/2/98; accepted 6/2/98.

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[1] This work was supported by NIH Grants RO1 CA 67409 and RO1 CA 72160, by American Cancer Society Grant FRA-451 (to S. M.), by an Advanced Research Training Award from the American Digestive Health Foundation (to W. M. G.), and by NIH Grants P30 CA 43703 and T32 CA 59366 (to Case Western Reserve University). S. M. is an investigator in the Howard Hughes Medical Institute.

[2] To whom requests for reprints should be addressed, at UCRC 2, Room 200, Ireland Cancer Center, 11001 Cedar Road, Cleveland, OH 44106.

[3] The abbreviations used are: TGF-β, transforming growth factor-β; RII, TGF-β type II receptor; MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colon cancer; MMR, mismatch repair.

Table 1 *Clinical and pathological features of colonic adenomas analyzed for TGF-β RII mutations in the BAT RII tract and MSI*

The adenomas are subdivided into those demonstrating microsatellite stability or MSI. The number of cases in each group represents the number of adenomas analyzed. Some patients had more than one adenoma identified in their colons.

	All cases (n = 73)	MSS[a] (n = 53)	MSI (n = 20)	MSI/wild-type *RII* (n = 13)	MSI/mutant *RII* (n = 7)
Patient characteristics					
Mean age (yr)	64	65	62	74	75
Age range (yr)	39–90	39–84	48–90	48–87	64–87
M:F ratio	41:16	31:7	10:9	4:5	4:3
Histology					
Tubular adenomas	21	16	5	5	0
Tubulovillous adenomas	9	7	2	2	0
Villous adenomas	4	4	0	0	0
Adenoma + focal high-grade dysplasia	17	12	5	4	1
Adenoma adjacent to adenocarcinoma	22	14	8	2	6
Location					
Proximal colon	66	48	18	12	6
Distal colon	7	5	2	1	1
TGFβ-*RII* mutation status[b]					
Wild type	66	53	13	13	0
Mutant	7	0	7	0	7

[a] MSS, microsatellite stability.
[b] $P < 0.005$ by χ^2 analysis.

CATTCAACATTTTTAACCC-3′ for *BAT26* and 5′-ACAACCCTGCTTTT-GTTCCT-3′ and 5′-GTAGAGCAAGACCACCTTG-3′ for *BAT40*, and for *D2S123*, *D2S147*, and *D10S197*, MapPairs (Research Genetics) were used as primers. Marker loci were PCR-amplified for 30–35 cycles using one ^{32}P-labeled primer and one unlabeled primer. Reaction products were resolved on a 6% LongRanger polyacrylamide (FMC Bioproducts)-7 M urea gel and visualized by autoradiography.

TGF-β *RII* Mutation Assay. A 73-bp region of the *RII* gene (nucleotides 665–737) was amplified from genomic DNA with ~10 ng of ^{32}P end-labeled TA10-F1 primer (5′-CTTTATTCTGGAAGATGCTGC-3′) and 150 ng of reverse primer TA10-R1 (5′-GAAGAAAGTCTCACCAGG-3′) using 30 cycles at 95°C for 30 s, 55°C for 1 min, and 70°C for 1 min, followed by a 72°C 4-min final extension. The PCR products were separated by electrophoresis at 52°C on a 6% LongRanger polyacrylamide (FMC Bioproducts)-7 M urea gel and visualized by autoradiography.

RESULTS AND DISCUSSION

Seventy-three adenomatous colon polyps from 57 patients were identified from archival material. The majority of adenomas (64 of 73) were selected from the proximal (right) colon, which is the principal site affected by the MSI pathway of colon neoplasia (Table 1; Refs. 11 and 16). None of the adenomas studied came from patients with a known familial cancer syndrome. To further enrich the study for adenomas representing sporadic MSI rather than HNPCC, we additionally selected adenomas only from patients who were more than 35 years old at the time of adenoma diagnosis (10). Table 1 shows that 34 of the adenomas studied were tubular, tubulovillous, or villous adenomas, which account for the first temporal stages of colonic neoplasia. The remaining 39 adenomas demonstrated further neoplastic progression and included regions of either high-grade dysplasia or of frank colon carcinoma that had invaded across the muscularis mucosa. In these advanced cases, the distinct regions of adenoma, high-grade dysplasia, and carcinoma were microdissected to isolate each component for further analysis.

As we have described previously, a PCR-based assay was used to detect frameshift mutations within the microsatellite-like coding region polyadenine repeat in the TGF-β *RII* (*BAT-RII*; Refs. 6 and 17).

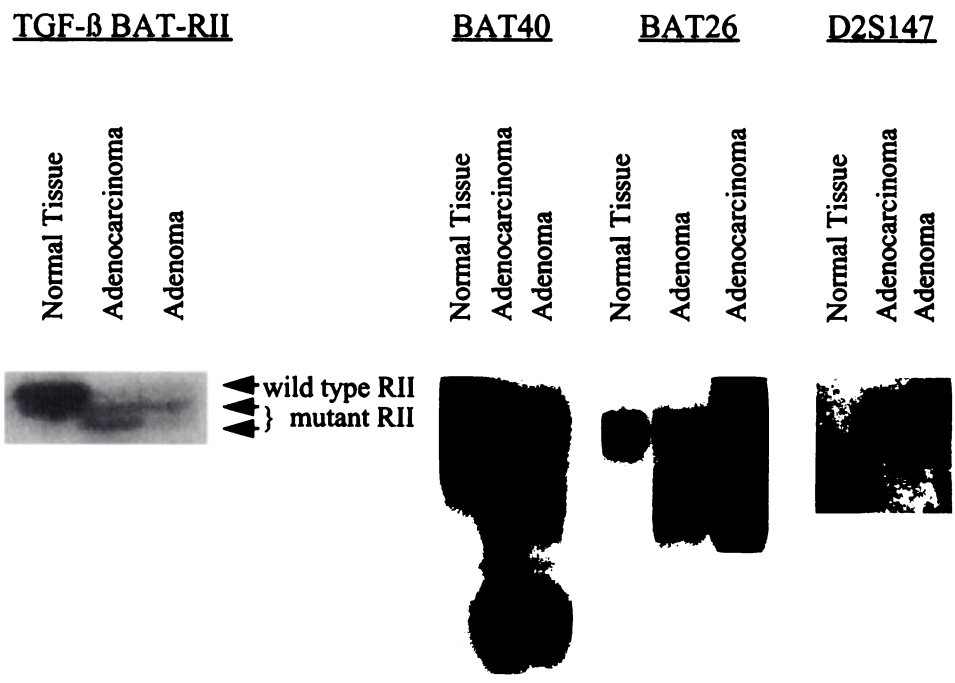

Fig. 1. Results from assays for TGF-β *BAT-RII* mutations and MSI in case 70, a representative case of an adenoma with a region of invasive adenocarcinoma. The TGF-β *BAT-RII* assay shows progressive loss of the wild-type TGF-β *RII* allele in the adenoma and associated adenocarcinoma. Assays for MSI are noted by the locus analyzed (*BAT40*, *BAT26*, or *D2S147*) and show MSI in both the adenoma and adjacent adenocarcinoma.

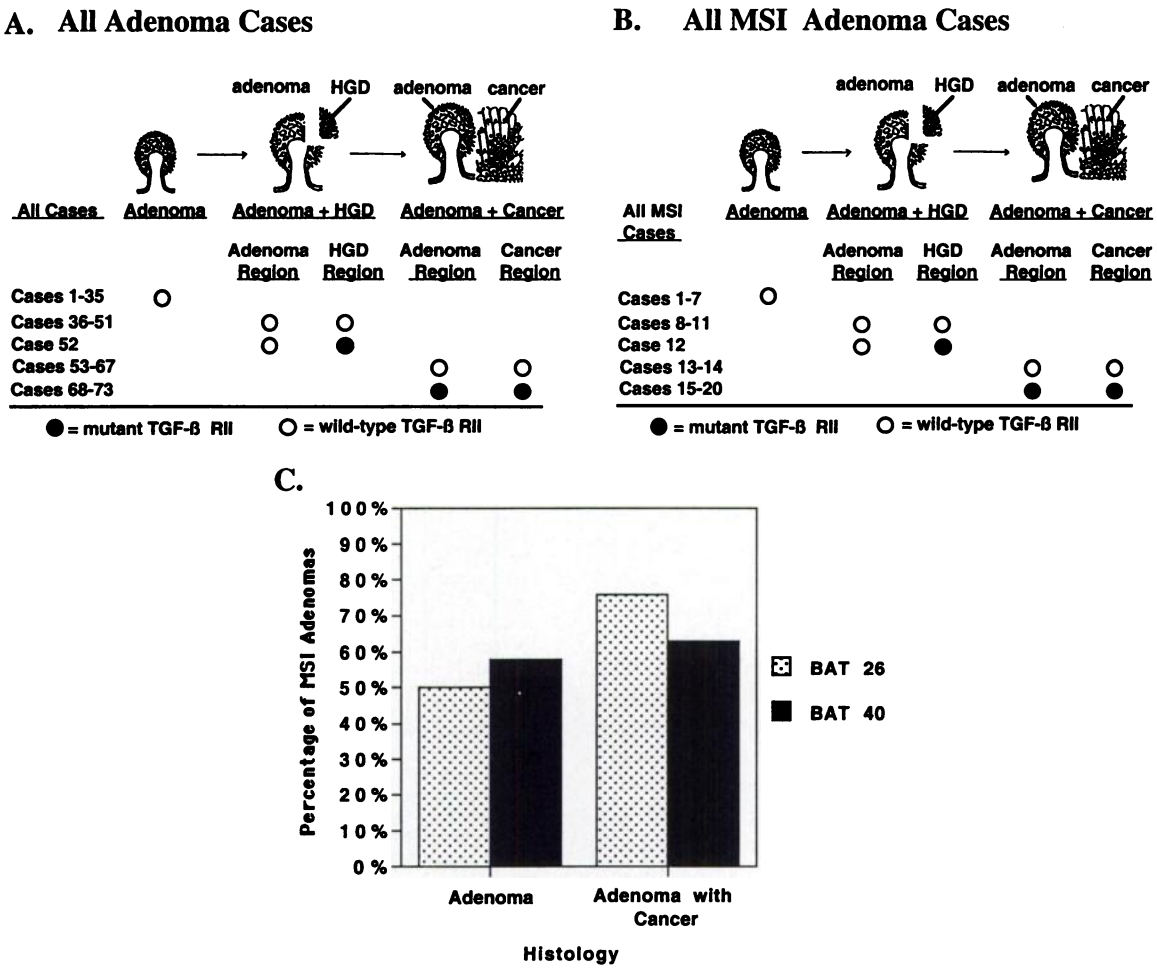

Fig. 2. *A*, a schematic representation of the incidence of TGF-β *BAT-RII* mutations in colonic adenomas. ○, cases with wild-type *RII*; ●, cases with *BAT-RII* mutations. Cases 1–35 are tubular, tubulovillous, or villous adenomas that have wild-type TGF-β *RII*. Cases 36–51 are adenomas with focal high-grade dysplasia (*HGD*) that also have wild-type TGF-β *RII* in the adenomatous tissue and dysplasia. Case 52 is an adenoma with high-grade dysplasia that shows a wild-type *RII* gene in the adenomatous tissue but a mutant TGF-β *RII* gene in the dysplasia only. Cases 53–73 are adenomas with regions of invasive adenocarcinoma. Six of these cases show TGF-β *RII* mutations in both the adenoma and adenocarcinoma. *B*, schematic representation of the incidence of TGF-β *BAT-RII* mutations in those colonic adenomas shown in *A* that additionally demonstrated MSI. *C*, incidence of MSI at the *BAT26* and *BAT40* loci in the MSI benign adenomas (*Adenoma*) compared to the MSI adenomas with regions of invasive adenocarcinoma (*Adenoma with Cancer*). *A* and *B* were modified from Bresalier, R. S., and Kim, Y. S. Malignant neoplasms of the large intestine. *In*: M. Feldman, M. H. Sleisenger, and B. F. Scharschmidt [eds.], Sleisenger and Fordtran's Gastrointestinal and Liver Disease, Ed. 6, p. 1914. Philadelphia: W. B. Saunders Company, 1998. Reprinted with permission.)

Fig. 1 demonstrates the normal-sized PCR fragment of the *BAT-RII* region amplified from one individual's normal colonic mucosa. In contrast, in a single late-stage colonic neoplasm from this individual, both regions of adenoma and carcinoma demonstrate complete loss of the wild-type *BAT-RII* signal and the acquisition of two mutant *RII* alleles inactivated by one and two base deletions, respectively, within the *BAT-RII* tract. Among the 73 adenomatous lesions studied, 7 demonstrated *BAT-RII* frameshift mutations. As shown in Fig. 2*A*, *BAT-RII* mutations were exclusively detected in late-stage, highly progressed adenomas. In six of the seven instances, *BAT-RII* mutations were detected in adenomas that contained regions that had progressed to frankly invasive adenocarcinoma. As shown in Fig. 2*A*, in each of these six instances the *BAT-RII* mutations were detected in both regions of residual adenoma as well as in the adenocarcinoma. In one additional case, a *BAT-RII* frameshift mutation was identified in a region of high-grade dysplasia, but only wild-type *RII* alleles were detected in the surrounding simple adenoma tissue. *RII* mutation is, thus, tightly temporally correlated with the adenoma-to-carcinoma transition. This is consistent with previous findings from our group demonstrating that restoration of wild-type *RII* abolishes the tumorigenic competence of a *RII* mutant colon cancer cell line (14). Several hypotheses may explain our observation that, in advanced colon adenomas bearing regions of frankly invasive carcinoma, the *RII* mutations were detected within both adenoma and carcinoma. Potentially, *RII* mutation might, in these adenomas, inactivate only one *RII* allele, whereas both *RII* alleles may be mutant in the adjacent regions of carcinoma. Alternatively, "adenomatous" regions of these compound neoplasms might be composed of fully transformed cells that are competent to invade across the muscularis mucosa but that retain a slightly more differentiated morphology when surrounded by normal colonic epithelium. Finally, full malignant transformation of colon epithelial cells may require mutation of both *RII* and an additional target gene. In this last model, the tight correlation of *RII* mutation with the adenoma-to-carcinoma transition would suggest that mutation of the second target gene must occur nearly simultaneously with or rapidly following the mutations in *RII*. Although we have noted colon cancers in which *RII* is inactivated by mutations outside the *BAT-RII* tract, these mutations appear to be infrequent and would not be expected to alter our conclusions above (5, 6).

Our previous studies demonstrated *BAT-RII* mutations exclusively in the subset of colorectal cancers that arise in concert with MSI. Accordingly, we presumed it likely that the seven neoplasms in which we detected *BAT-RII* mutations would demonstrate MSI. To further examine the relationship between MSI and *BAT-RII* mutations in these colon adenomas, we characterized the 73 colon neoplasms for MSI. Fig. 1

shows for one individual the microsatellite fingerprint in normal colonic mucosa compared with the diagnostic pattern of microsatellite shifts demonstrating MSI of the adenomatous and carcinomatous regions of his colon neoplasm. As shown in Table 1, MSI was detected in 20 of the 73 colon adenomas, including 8 highly progressed neoplasms containing regions of frankly invasive carcinoma. In all eight cases, MSI was detected in both the adenomatous and the carcinomatous components of the neoplasms. As shown in Fig. 2*B*, six of the eight late adenomas demonstrated *BAT-RII* mutations. In each case *BAT-RII* mutations were present within both regions of adenoma and carcinoma. Thus, 75% of late-stage MSI adenomas demonstrated *BAT-RII* mutations. This incidence of mutations is statistically indistinguishable from our previous finding of *BAT-RII* mutations present in 90% of frank MSI+ colon cancers (6). These findings confirm that *BAT-RII* mutations are a common mechanism for neoplastic progression of colon neoplasms with MSI. The temporal clustering of these mutations at the adenoma-to-carcinoma transition supports the hypothesis that these mutations subserve a distinct biological functional during the neoplastic transformation of colon epithelium. As shown in Fig. 2*B*, of 12 early-stage adenomas with MSI, only 1 case demonstrated a *BAT-RII* mutation, and this mutation was confined to a region of high-grade dysplasia arising within the adenoma. Therefore, the association of *BAT-RII* mutations with late colon adenomas is not due to any paucity of early stage MSI adenomas in this study. Further support for this conclusion is offered by the observation that, in three individuals who had both simple and progressed MSI adenomas, only the progressed adenomas, which all contained regions of frankly invasive carcinoma or high-grade dysplasia, showed mutant TGF-β *RII*. MSI is likely present in early adenomas, and the development of MSI thus precedes the mutational inactivation of *RII*. We suggest this is true of sporadic MSI adenomas arising outside the setting of HNPCC (10, 11). Consistent with the sporadic origin of the MSI adenomas characterized in this study is that the mean age of individuals with adenomas with MSI and microsatellite stability are essentially the same (62 *versus* 65 years old, respectively; Table 1).

Colon neoplasms arising via the MSI pathway acquire unselected microsatellite shifts stochastically. Hence, the frequency of shifted microsatellites should increase as a neoplasm ages (18). Alternatively stated, adenomas with MSI are expected to have a lower frequency of microsatellite shifts than typical of carcinomas with MSI. This phenomenon was illustrated in one case of an adenoma containing a region of invasive adenocarcinoma in which we detected shifts in *BAT26*, *BAT40*, and *BAT-RII* in both the adenoma and carcinoma but detected shifts in microsatellite loci *D2S147* and *D10S197* only in the carcinoma. In accord with this model, in this study, we regarded adenomas shifted at either the *BAT26* or *BAT40* locus as arising via the MSI pathway. Consistent with this criteria, we observed shifts in one but not both of the loci (*BAT26* and *BAT40*) in four of the seven adenomas that demonstrated *BAT-RII* mutations, which, hence, most certainly arose via the MSI pathway. Compared with the 98% sensitivity that both *BAT26* and *BAT40* have in detecting MSI in colon cancer (6, 15), Fig. 2*C* shows that *BAT26* and *BAT40*, indeed, have lesser sensitivities, of 50 and 58%, respectively, for detecting MSI in early adenomas and intermediate sensitivities of 75 and 63% for detecting MSI in progressed adenomas. Although these findings support using a "relaxed" definition of MSI in studies of adenomas and other early neoplasms, this issue does not directly affect our principal conclusion that *BAT-RII* mutations are associated nearly exclusively with adenomas in the midst of the adenoma-to-carcinoma transition.

In summary, colon cancer cell lines are commonly resistant to TGF-β-mediated growth inhibition (3, 5). In 90% or more of colon cancers with MSI, TGF-β resistance is due to mutations in the *RII* component of the TGF-β receptors. In a small number of other colon cancers, TGF-β resistance may be due to mutation of *Smad2* or *Smad4* (7–9), which have been demonstrated to play a role in TGF-β signal transduction. This study demonstrates that, in the MSI pathway of carcinogenesis, *RII* mutations first arise nearly exclusively in adenomas in the midst of the transition to invasive colon cancer. *RII* mutation, thus, appears to inactivate a late checkpoint, the absence of which is required for the acquisition of the ability of transformed colon cells to demonstrate frank invasiveness into normal colon tissues. Further characterization of the TGF-β signal transduction pathway and the genes regulated by this pathway should further illuminate the physiology of this tumor suppressor checkpoint.

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Cancer Res 1998;58:3101-3104.