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Advances in Brief

Mutational Inactivation of Transforming Growth Factor β Receptor Type II in Microsatellite Stable Colon Cancers[1]

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Abstract

We previously demonstrated that mutational inactivation of transforming growth factor β type II receptors (RIIs) is very common among the 13% of human colon cancers with microsatellite instability. These mutations principally cluster in the *BAT-RII* polyadenine sequence repeat. Among microsatellite stable (MSS) colon cancers, we now find that non-*BAT-RII* point mutations inactivate RII in another 15% of cases, thus doubling the known number of colon cancers in which *RII* mutations are pathogenetic. Functional analysis confirms that these mutations inactivate RII signaling. Moreover, another 55% of MSS colon cancers demonstrate a transforming growth factor β signaling blockade distal to RII. The transforming growth factor β pathway and RII in particular are major targets for inactivation in MSS colon cancers as well as in colon cancers with microsatellite instability.

Introduction

TGF-β[4] inhibits the growth of epithelial cells in general (1, 2) and can inhibit growth and/or induce apoptosis in nontransformed colon epithelial cells (3, 4). TGF-β signaling is transduced by a heteromeric receptor complex composed of type I and type II components, both of which are serine/threonine-directed kinases (5). A role for *RII* as a human colon cancer tumor suppressor gene was demonstrated by the discovery of inactivating *RII* mutations in colorectal cancers that show MSI due to defects in DNA mismatch repair (6–8). These cancers with MSI account for 13% of all colon cancers (6, 7, 9). Furthermore, the restoration of wild-type *RII* in cell lines from colon cancers with MSI abolishes their tumorigenicity in athymic mice (10). *RII* mutations in colon cancers with MSI usually result in frameshifts clustered in a naturally occurring 10-bp microsatellite-like polyadenine tract in the 5′ coding half of the gene (*BAT-RII*; Refs. 7 and 8). In a few colon cancers with MSI, inactivation of one of the *RII* alleles occurs via non-*BAT-RII* mutations that alter the RII kinase domain (7, 9, 11), demonstrating an underlying selective advantage for RII inactivation, irrespective of whether this occurs via *BAT-RII* or non-*BAT-RII* mutational events. We hypothesized that the *RII* mutation should function similarly as a tumor suppressor gene in MSS colon cancers. Accordingly, we examined the *RII* sequence in 19 MSS colorectal cancer cell lines. These Vaco cell lines have been matched to antecedent tumor and normal tissues and have been extensively characterized as a model for human colon cancer (7, 12–14). Three of the 19 cell lines were shown to express mutant-only *RII* transcripts. Functional analysis showed that the *RII* mutations inactivated RII signaling in each case. Moreover, an additional 11 of these cell lines demonstrated a loss of TGF-β responsiveness, apparently through post-RII signaling defects. Therefore, TGF-β signaling in general and RII in particular are targets for inactivation in MSS colon cancers as well as in colon cancers with MSI.

Materials and Methods

Cell Lines and Primary Tumors. The establishment of the panel of the Vaco colon cancer cell lines has been described previously (7, 12). The Vaco8-2 cell line was established from a stage IV cecal colon adenocarcinoma from a 56-year-old male. Vaco400 was established from a liver metastasis from a 54-year-old man. Vaco410 was derived from a stage IV colon adenocarcinoma metastasis in the right lobe of the liver of a 35-year-old woman. These cell lines were maintained as described previously (12). Genomic DNA from the original paraffin-embedded formalin-fixed tumor blocks was extracted as described previously (15). DNA from matched normal tissue for each colon cancer was obtained when available.

TGF-β Growth Inhibition Assay. The colon cancer cell lines were plated at clonogenic density (100–500 cells/well in 24-well plates for adherent cell lines and 1000–5000 cells/well in 6-well plates for collagen-dependent adherent cell lines) and treated with TGF-β1 (10 ng/ml) 3 h after plating. The cell lines were grown until discrete microscopic colonies appeared (usually for 7–10 days), and then the number of colonies in each well was manually counted. Mean and SEs of the means were calculated from experiments performed in triplicate wells and repeated in at least nine independent determinations.

RT-PCR Amplification and Sequencing of RII. RNA from the cell lines was prepared by extraction with guanidine isothiocyanate (7). RT-PCR and cloning of the reaction products and manual sequencing of the cloned DNA were performed as described previously (7), or the reaction products were sequenced by automated sequencing using a ABI 377 DNA Sequencer. For amplification of *RII* from genomic DNA, sense primer 1871 (5′-GGTGTGTGAGACGTTGACTGAGTG-3′) was paired with antisense primer 2001 (5′-AATCTTCTCCTCCGAGCAGCTC-3′) for 30 cycles of 95°C for 30 s, 58°C for 1 min, and 70°C for 1 min. All *RII* mutations were confirmed to be present in at least two independently amplified PCR reactions compared with the reference sequence (GenBank accession number M85079).

TGF-β Signaling Analysis. The cell lines were transiently transfected following the manufacturers' protocols using Lipofectin (Life Technologies, Inc., Gaithersburg, MD), Superfect (Qiagen), or FuGENE (Boehringer Mannheim) with a TGF-β-responsive firefly luciferase reporter plasmid (p3TP-Lux; Ref. 16) and an internal control reporter plasmid containing cDNA encoding *R. reniformis* luciferase under a thymidine kinase promoter (pRL-TK) or a

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[4] The abbreviations used are: TGF-β, transforming growth factor β; RII, TGF-β receptor type II; MSI, microsatellite instability; MSS, microsatellite stable; RT-PCR, reverse transcription-PCR.

cytomegalovirus promoter (pRL-CMV; Promega, Madison, WI). The p3TP-Lux plasmid was kindly provided by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center and Howard Hughes Medical Center, New York, NY). After transfection, the cell lines were exposed to TGF-β1 (10 ng/ml) for 72 h and then assessed for reporter activity. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System following the protocol included with this kit (Promega). The samples were assayed on a Turner Dual Injector Luminometer (Promega) or a MLX Multiplate Luminometer (Dynex Technologies).

Retroviral Transduction. The cell lines were infected with the MFG-RII or MFG-CAT retroviruses (17). Cells were plated in 6-well plates at a density of 200,000 or 400,000 cells/well; on the following day, they were incubated in MFG-RII or MFG-CAT viral supernatant produced by a virus producer cell line and polybrene (4 μg/ml) for 4 h. The cells then were rinsed three times in PBS. G418 (600 μg/ml) was added 2 days after the viral infection. TGF-β1 (10 ng/ml) was added to designated subsets of the infected cell lines 3 h after infection to assess for reconstitution of TGF-β-induced growth inhibition. The growth inhibition assays were performed as described above. All of these experiments were performed in triplicate and repeated at least three times.

Results and Discussion

MSS Colon Cancers Are Commonly TGF-β Resistant. We initially determined whether TGF-β growth-inhibitory responses were intact in 19 colon cancer cell lines that had been previously determined to be MSS (7). TGF-β growth inhibition was assayed by determining the ability of TGF-β1 to inhibit the colony formation of cells plated at clonogenic density. Cell lines in which TGF-β1 (10 ng/ml) reduced colony formation at 7–10 days after plating by >25% were considered to be responsive to TGF-β-mediated growth inhibition. Five cell lines (26%) were determined to demonstrate sensitivity to TGF-β-mediated growth inhibition, with TGF-β-mediated suppression of colony formation ranging from 42–79%. However, 74% (14 of 19) of the MSS cell lines were found to be resistant to the growth-inhibitory effects of TGF-β.

RII Mutations Detected in MSS Colon Cancers. Ninety percent of colon carcinoma cell lines with MSI demonstrate both TGF-β resistance and *BAT-RII* mutations that inactivate the RII receptor (7, 9). Accordingly, we initially examined the MSS colon cancers for *BAT-RII* mutations using our previously described assay (8, 9). Consistent with our prior results, no *BAT RII* mutations were detected in any of these cell lines (7). Therefore, we determined the complete *RII* cDNA sequences in RT-PCR cDNA pools amplified from each of the 14 MSS cell lines that demonstrated TGF-β resistance. In three cell lines (15%), only mutant *RII* sequences were obtained, and no wild-type *RII* was expressed. In the remaining cell lines, *RII* was expressed and was wild-type in sequence. *RII* mutations were previously demonstrated to be ubiquitous among the approximately 13% of colon cancers that show MSI (7, 9, 18). These new findings therefore double the number of colon cancers in which *RII* mutations are pathogenetic to approximately 28% of all colon adenocarcinomas and show that such *RII* mutant cases occur in both the MSI and MSS subtypes.

The mutations detected in MSS cell lines Vaco8-2, Vaco400, and Vaco410 are located within conserved regions of the kinase domain of the *RII* gene (Vaco8-2, Vaco410, and Vaco400 cells) or in the 5′ extracytoplasmic portion of the receptor (Vaco400 cells). In Vaco8-2 cells, a missense mutation (GAC→AAC) changed an aspartic acid to an asparagine at codon 522 (Fig. 1; Ref. 19), altering the charge of a residue within subdomain XI of the serine/threonine kinase domain of the receptor. In Vaco410 cells, a missense mutation (CGT→CAT) changed an arginine to a histidine at codon 528 (Fig. 1; Ref. 19). This arginine, which is also within kinase subdomain XI, is strictly conserved among all serine/threonine protein kinases (20). In Vaco400 cells, two mutations were observed in the pooled cDNA products; both were apparently heterozygous (Fig. 1). Sequencing individual RT-PCR cDNA clones revealed that this was due to separate mutations present on each of the two expressed Vaco400 alleles. One Vaco400 allele carried a missense mutation (TAT→GAT) at codon 470 that changed a tyrosine to an aspartic acid (19) and altered the charge of an amino acid in subdomain IX of the receptor kinase domain. The second Vaco400 allele carried a missense mutation (AAA→ACA) at codon 52 that changed a lysine to a threonine in the extracytoplasmic region of the receptor.

To confirm that these base changes did not merely arise during the establishment of the cell lines, we analyzed genomic DNA from the tumors from which Vaco8-2, Vaco400, and Vaco410 were established. In each case, the *RII* mutations were demonstrated to be present in the antecedent tumors from which the cell lines were derived. Normal tissue was also available from individuals matched to Vaco410 and Vaco400. In both instances, only wild-type *RII* sequences were demonstrated in the normal tissues, confirming that in these tumors, *RII* mutations arose somatically and were selected for during carcinogenesis.

Kinase domain mutations in *RII* have previously been observed in occasional colon cancers with MSI both by us and by other investigators (9, 11) and have also been noted in two squamous cell carcinomas of the oropharynx (21) and in one T-cell lymphoma (22). The selection of these Vaco colon cancer mutations during tumorigenesis, their location within *RII*, and the nature of the amino acid changes produced suggested that these mutations likely inactivated the TGF-β receptor and induced the TGF-β resistance observed in the Vaco cell lines. Of note, *RII* mutations were not observed in an investigation of

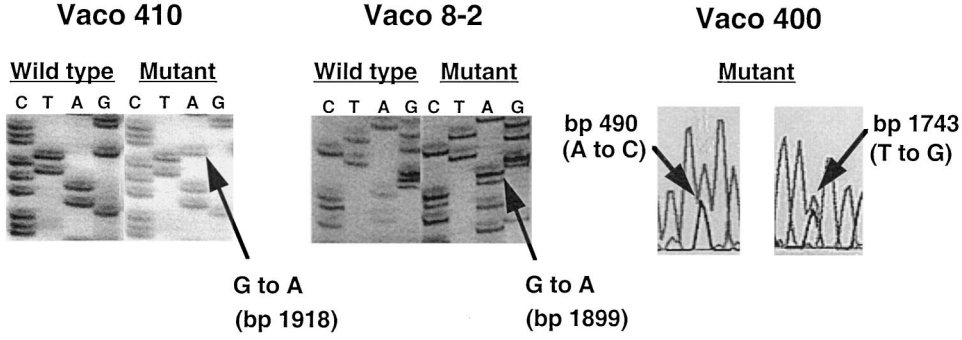

Fig. 1. Point mutations of TGF-β *RII* in colon cancer cell lines Vaco410, Vaco8-2, and Vaco400. *RII* cDNA was PCR-amplified in two overlapping pieces, cloned into the pCRII vector (Invitrogen), and then sequenced manually or by ABI automated sequencing. Vaco410 has a homozygous missense mutation in exon 7 at bp 1918 (G→A) that changes codon 528 from Arg to His. Vaco8-2 has a homozygous missense mutation at bp 1899 (G→A) that changes codon 522 from Asp to Asn. Vaco400 has two heterozygous missense mutations that are on opposite alleles. One mutation is located at bp 490 and changes codon 52 from Lys to Thr, and the other mutation is at bp 1743 and changes codon 470 from Tyr to Asp. The mutations in Vaco400 (at codon 470), Vaco410, and Vaco8-2 all affect the kinase domain of the receptor. The mutation at codon 52 in Vaco400 affects the extracytoplasmic portion of RII.

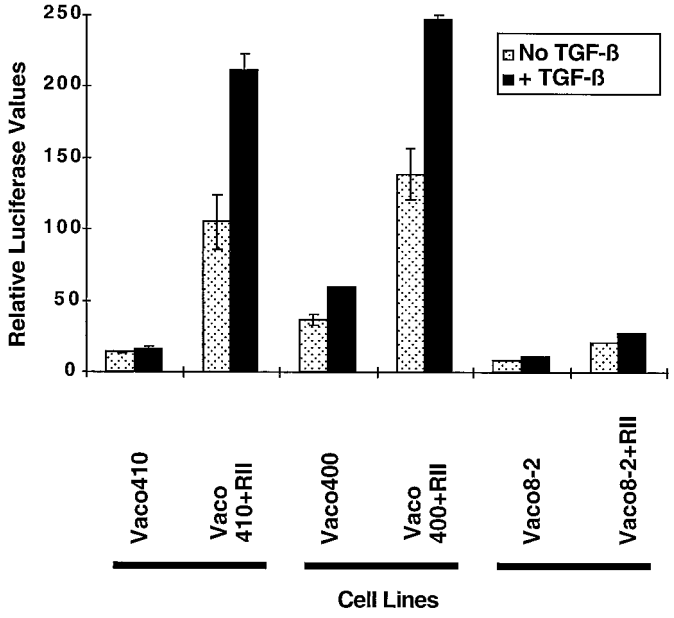

Fig. 2. Restoration by *RII* of TGF-β-stimulated transcriptional responses. Relative luciferase activity from TGF-β-responsive reporter plasmid 3TPLux in cell lines Vaco410, Vaco400, and Vaco8-2 after transient transfection with pRC/CMV *RII* is shown. The cells were grown for 2 days after transfection with or without TGF-β1 (10 ng/ml). Vaco410 and Vaco400 demonstrate both a basal and TGF-β-inducible increase in 3TPLux activity after transfection with *RII*. The basal induction in Vaco410 and Vaco400 is presumably secondary to autocrine TGF-β produced by these cell lines. Vaco8-2 shows no increase in 3TPLux activity after *RII* transfection with or without the addition of TGF-β1. The luciferase activity was normalized to the activity of a cotransfected control vector, pRL-TK or pRL-CMV, that contains the distinguishable *R. reniformis* luciferase.

a different set of colon cancers studied by single-strand conformational polymorphism; this contrasting result is most likely a consequence of the lower sensitivity of single-strand conformational polymorphism compared to sequencing (11).

RII Mutations Inactivate TGF-β Receptor Signaling. To confirm that the *RII* mutations detected in the MSS colon cancers inactivated the receptor, we determined the ability of wild-type *RII* to restore TGF-β-mediated responses in these cell lines. We initially assayed the ability of wild-type *RII* to restore TGF-β-mediated transcriptional responses as assayed by the TGF-β-responsive firefly luciferase activity encoded by the reporter construct p3TP-Lux (16). p3TP-Lux was transiently transfected into these cell lines accompanied by either *RII* expression vector, pRC/CMV-TGF-βRII, or control DNA, and p3TP-Lux-mediated luminescence was determined in the presence and absence of exogenous TGF-β1. Each determination was corrected for transfection efficiency by assaying for the luminescence from a cotransfected control construct, pRL-TK or pRL-CMV (Promega), a plasmid that expresses *R. reniformis* luciferase activity that is easily discriminated from firefly luciferase activity.

Consistent with the findings that Vaco8-2, Vaco400, and Vaco410 were resistant to TGF-β-mediated growth inhibition, each of these cell lines demonstrated low basal activity of p3TP-Lux and no further response of the reporter to added TGF-β1 (Fig. 2). In Vaco410 and Vaco400 cells, cotransfection of wild-type *RII* augmented the basal p3TP-Lux activity. p3TP-Lux activity increased further after the addition of exogenous TGF-β1, such that total the 3TP-Lux output reached 5–10-fold over baseline (Fig. 2). The increase in p3TP-Lux activity due to the introduction of wild-type *RII* likely reflects the activation of the restored TGF-β signaling pathway by the autocrine TGF-β produced by these cell lines.[5] The responsiveness of p3TP-Lux to exogenous TGF-β1 clearly establishes that introducing wild-type *RII* restores TGF-β-mediated signaling in these cell lines. Thus, the *RII* mutations in Vaco410 and Vaco400 directly account for the TGF-β resistance of these cell lines. As discussed below, we hypothesized that the Vaco8-2 *RII* mutation also inactivated receptor signaling, but that additional progression events in this cell line interfered with the ability to reconstitute TGF-β signaling by a single gene alone.

Tumor Suppressor Activity of Wild-Type RII in MSS Colon Cancer. To assess the potency of the TGF-β tumor suppressor pathway inactivated in *RII* mutant MSS colon cancers, we determined the ability of wild-type *RII* to inhibit colony formation in *RII* mutant cancer cells. Colon cancer cells were plated at a concentration selected to achieve clonogenic density after G418 selection and transduced with MFG-RII, a replication-incompetent retrovirus encoding wild-type *RII,* or an equal titer of a control MFG-CAT retrovirus, and then colonies arising in the presence or absence of TGF-β1 were counted and compared. Noninfected cells were removed from the assay by selection of the cells in G418. As expected, TGF-β1 had no colony suppression activity in any of the cell lines infected with the control MFG-CAT virus (Fig. 3). In contrast, 90% of colonies were suppressed in both Vaco400 and Vaco410 cells that were transduced with wild-type *RII* and treated with TGF-β1 (Fig. 3). Thus, *RII* is revealed as a potent suppressor gene in these cell lines, and the *RII* mutations in these tumors are again confirmed as the cause of their resistance to TGF-β growth inhibition. Similar to the inability of wild-type *RII* in Vaco8-2 to restore TGF-β-mediated transcriptional responses, we observed that wild-type *RII* also did not restore TGF-β-mediated growth inhibition in Vaco8-2. RT-PCR and Western blot analysis confirmed that after MFG-RII infection in Vaco8-2, wild-type *RII* cDNA was expressed, and RII protein expression was increased to levels similar to those obtained in MFG-RII-infected Vaco400 and Vaco410. Parenthetically, we noted that because G418-resistant Vaco8-2 colony numbers were the same in MFG-CAT- and MFG-RII-transduced Vaco8-2, there was no apparent nonspecific toxicity of the MFG-RII virus relative to the MFG-CAT control. Thus, the partial reduction of Vaco400 and Vaco410 colony numbers by the MFG-RII retrovirus alone is likely due to the autocrine TGF-β produced by these cell lines.

Mutation of Both RII and Smad4 in Vaco8-2. Because wild-type *RII* did not restore TGF-β-mediated signaling in the Vaco8-2 cell line, the functional properties of the Vaco8-2 *RII* mutant were studied by

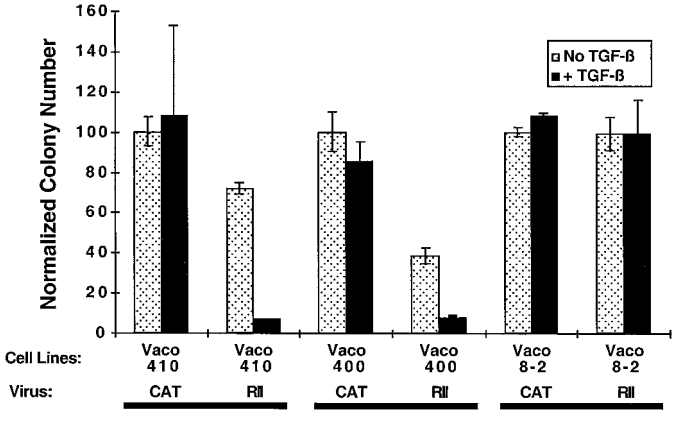

Fig. 3. Suppression of colony-forming activity by wild-type *RII*. The cell lines were plated at a concentration to yield clonogenic density after G418 selection, infected with a RII retrovirus, and then assessed for colony formation 7–10 days after being grown in the presence or absence of exogenous TGF-β1 (10 ng/ml). Reconstitution of *RII* suppresses the number of colonies formed in Vaco400 and Vaco410. This suppression is significantly augmented by exogenous TGF-β. Vaco8-2 shows no suppression of colony formation after *RII* reconstitution. MFG-CAT, a MFG retrovirus encoding CAT and a neomycin resistance gene, was used to control for nonspecific effects of the retrovirus.

[5] S. Markowitz and M. G. Brattain, unpublished data.

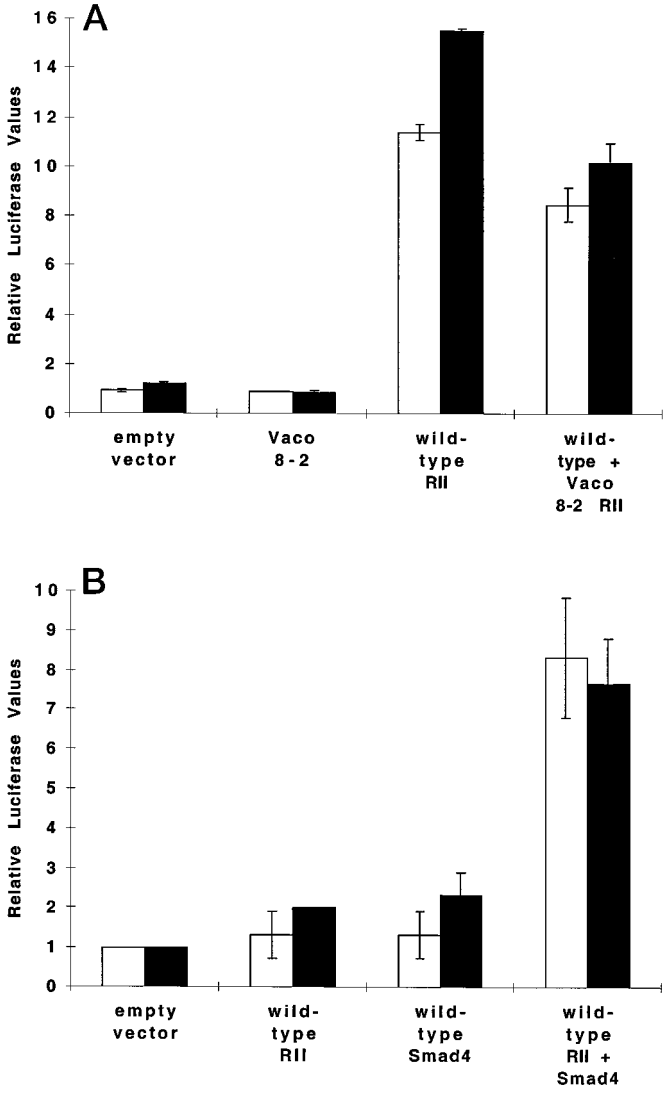

Fig. 4. *A*, transcriptional activity of wild-type and mutant *RII* transfected into Hct116. The relative luciferase activity from the 3TPLux reporter plasmid in the RII-null cell line Hct116 is shown after transfection with wild-type *RII* cDNA in pRC/CMV (*wild-type RII*), Vaco 8-2 mutant *RII* cDNA in pRC/CMV (*Vaco8-2*), or an empty pRC/CMV control vector (*empty vector*). Transfections were done with (■) and without (□) treatment with TGF-β1 (10 ng/ml) for 48 h. Equal amounts of expression vectors for wild-type *RII* and Vaco8-2 mutant *RII* were also mixed and cotransfected (*wild-type + Vaco8-2 RII*). Transfections were normalized relative to the activity of a cotransfected pRL-TK or pRL-CMV vector. *B*, TGF-β induced transcriptional responses in Vaco8–2. The relative luciferase activity from 3TPLux in Vaco8-2 is shown after transfection with wild-type *RII*, wild-type *Smad4*, or a combination of both wild-type *RII* and *Smad4*. Induction of 3TPLux activity is observed only after the transfection of *RII* and *Smad4*.

cloning this mutation into a cDNA expression vector and transiently transfecting the Vaco8-2 *RII* into the RII-deficient cell line Hct116 (7). As shown in Fig. 4*A*, transient transfection of wild-type *RII* restored TGF-β signaling in Hct116 as assayed by the p3TP-Lux reporter. In contrast, the Vaco8-2 *RII* was inactive in restoring RII function in Hct116. Hence, the Vaco8-2 *RII* mutation that was selected for during Vaco8-2 tumorigenesis is sufficient to inactivate the TGF-β receptor and to abolish TGF-β signaling.

We initially considered the hypothesis that the Vaco8-2 *RII* mutation might have dominant negative activity, rendering the cell line resistant to the addition of a wild-type allele. However, cotransfecting the Vaco8-2*RII* and wild-type *RII* expression vectors revealed, at best, a borderline inhibition of wild-type RII by the Vaco8-2 mutant. (Fig. 4*A*); accordingly, we hypothesized that in Vaco8-2, a separate event had further inactivated the TGF-β signaling pathway.

The binding of TGF-β to its receptor activates a heteromeric complex of Smad2-Smad3-Smad4 transcription factors to translocate to the nucleus (23, 24). Previously, we and others have reported that mutations of *Smad2* and *Smad4* are present at a low frequency in some human colon cancers (13, 14, 25, 26). Accordingly, we examined the possibility of Smad inactivation as a separate event in the Vaco8-2 TGF-β signaling pathway. Whereas neither *Smad4* nor *RII* transfected singly into Vaco8-2 triggered the 3TP-Lux reporter, cotransfection of *RII* and *Smad4* triggered a 5–10-fold p3TP-Lux response, thus suggesting that the inactivation of *RII* and *Smad4* accounted for two separate defects in the Vaco8-2 TGF-β signaling pathway (Fig. 4*B*). Examination of the Vaco8-2 *Smad4* gene revealed that neither *Smad4* exon 1 nor exon 2 could be amplified from Vaco8-2 genomic DNA. Thus, biallelic deletion of *Smad4* was indeed a second genetic event in the Vaco8-2 cell line TGF-β pathway. Due to the small amounts of residual normal tissue remaining in even the microdissected tumor antecedent to the Vaco8-2 cell line, we could not establish whether *Smad4* loss occurred in the predecessor Vaco8-2 tumor or only after establishment of the Vaco8-2 cell line. Nonetheless, it is intriguing to speculate that the presence of mutations in both the *RII* and *Smad4* genes in the Vaco 8-2 cell line, although rare, seems to suggest that RII and Smad4 may have some functions that are distinct from one another.

In summary, multiple lines of evidence now suggest that the TGF-β pathway is a potent tumor suppressor of human colorectal carcinogenesis. We have previously determined that among the 13% of human colon cancers of the MSI subtype, nearly all have inactivating *RII* frameshift mutations clustering at the *BAT-RII* tract (7, 9). Data from the Vaco colon cancer cell lines now suggest that among MSS human colon cancers, an additional 15% of tumors also bear inactivating *RII* mutations, and that restoring wild-type *RII* to these tumors is potently growth suppressive. Hence, *RII* mutations play a pathophysiological role in approximately 30% of human colon cancers, and *RII* gene therapy may have a future role in the treatment of these tumors. Analysis of TGF-β sensitivity in the Vaco colon cancer cell line panel further suggests that an additional 55% of human colon cancers demonstrate functional inactivation of TGF-β-mediated growth inhibition. Furthermore, these cancer lines have lost TGF-β-mediated transcriptional responses as well.[6] Presumptively, these additional cancers bear defects in the TGF-β signaling pathway at points distal to RII. We have previously shown that *Smad2* and *Smad4* mutations are present in only a small percentage of human colon cancers (13, 14, 27). However, the tumor-promoting activity of such downstream inactivation of TGF-β signaling is suggested by recent studies in murine models showing that heterozygous *Smad4* mutation promotes colon adenoma to carcinoma progression (28), and homozygous *Smad3* knockout leads directly to colon cancer (29). Study of additional mechanisms leading to the inactivation of TGF-β signaling in the remaining set of wild-type *RII* MSS colon cancers is currently ongoing.

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