The Bone Morphogenetic Protein Pathway Is Inactivated in the Majority of Sporadic Colorectal Cancers

LIUDMILA L. KODACH,* ELIZA WIERCINSKA,‡ NOEL F. C. C. DE MIRANDA,§ SYLVIA A. BLEUMING,‖ ALEX R. MUSLER,¶ MAIKEL P. PEPPELENBOSCH,# EVELIEN DEKKER,** GIJS R. VAN DEN BRINK,* CAREL J. M. VAN NOESEL,¶ HANS MORREAU,‖ DANIEL W. HOMMES,* PETER TEN DIJKE,‡ G. JOHAN A. OFFERHAUS,‡‡ and JAMES C. H. HARDWICK*

*Departments of *Gastroenterology, ‡Molecular Cell Biology, and §Pathology, Leiden University Medical Center, Leiden; Departments of ‖Experimental and Molecular Medicine, ¶Pathology, and **Gastroenterology, Academic Medical Centre, Amsterdam; #Department of Cell Biology, University Medical Center Groningen, University of Groningen, Groningen; and ‡‡Department of Pathology, University Medical Center, Utrecht, The Netherlands*

Background & Aims: **The finding of bone morphogenetic protein (BMP) receptor 1a mutations in juvenile polyposis suggests that BMPs are important in colorectal cancer (CRC). We investigated the BMP pathway in sporadic CRC.** ***Methods:*** **We investigated BMP receptor (BMPR) expression using immunoblotting and sequenced BMPR2 in CRC cell lines. We assessed the expression of BMPRs, SMAD4, and pSMAD1/5/8 in 72 sporadic CRCs using a tissue microarray and immunohistochemistry. We assessed the effect of reintroduction of wild-type BMPR2 on BMP pathway activity and the effect of wild-type or mutated BMPR2 3′ untranslated region (UTR) sequences on protein expression by attachment to pCMV-Luc.** ***Results:*** **BMPR2 and SMAD4 protein expression is abrogated in microsatellite unstable (MSI) and microsatellite stable (MSS) cell lines, respectively. BMPR2 3′UTR is mutated in all MSI and in none of the MSS cell lines. Mutant BMPR2 3′UTR sequences reduced luciferase expression 10-fold compared with wild-type BMPR2 3′UTR. BMPR2 expression is impaired more frequently in MSI CRCs than MSS (85% vs 29%; $P < .0001$) and shows a mutually exclusive pattern of impaired expression compared with SMAD4. Nine of 11 MSI cancers with impaired expression of BMPR2 have microsatellite mutations. The BMP pathway is inactivated, as judged by nuclear pSMAD1/5/8 expression, in 70% of CRCs, and this correlates with BMPR and SMAD4 loss.** ***Conclusions:*** **Our data suggest that the BMP pathway is inactivated in the majority of sporadic CRCs. In MSI CRC this is associated predominantly with impaired BMPR2 expression and in MSS CRC with impaired SMAD4 expression.**

The transforming growth factor (TGF)-β signaling pathway is believed to play a central role in colorectal cancer (CRC).[1] The TGF-β superfamily consists of the TGF-β, activin, and bone morphogenetic protein (BMP) subfamilies. TGF-β receptor 2 (TGFβR2) has long been considered the most frequently mutated gene in microsatellite unstable (MSI) cancers,[2] but how this leads to cancer is unclear with conditional TGFβR2 knockout from the colonic epithelium in mice showing no CRC phenotype.[3] Disturbances of the downstream mediators of TGF-β function, the SMADs, do have a CRC phenotype in mice,[4,5] and mutations of SMAD4 are frequently found in human CRC.[6] Signaling via SMAD4 is not exclusively activated by TGF-β but can also be activated via both the activin and BMP receptors. Recent work has shown very high rates of mutation of the activin receptor 2 (ACVR2)[7] in MSI CRC, but the possible involvement of the BMP pathway in sporadic CRC has received little attention.

Several recent findings suggest the involvement of BMPs in CRC. First, the mutations in SMAD4 frequently found in colon cancers may implicate not only TGF-β but also BMPs in colon cancer progression, because SMAD4 is central to both BMP and TGF-β signal transduction. Second, up to 50% of individuals with juvenile polyposis, an inherited syndrome with a high risk of developing CRC, carry germline mutations in either BMP receptor (BMPR) 1a or SMAD4 genes.[8,9] This is further supported by a transgenic mouse model of juvenile polyposis, the villin-noggin mouse in which BMP expression is completely abrogated, which also develops neoplasia.[10] Third, BMP acts as a tumor suppressor promoting apoptosis in mature colonic epithelial cells, and therefore perturbations in BMP signaling could lead to increased tumorigenesis.[11] Finally, conditional inactivation of BMPR2 in the intestine in mice leads to increased colonic epithelial proliferation and polyp formation.[12]

Abbreviations used in this paper: ACVR2, activin receptor 2; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor; CMV, cytomegalovirus; CRC, colorectal cancer; GFP, green fluorescent protein; MMR, mismatch repair; MSI, microsatellite unstable; MSS, microsatellite stable; PCR, polymerase chain reaction; TGF, transforming growth factor; TMA, tissue microarray; UTR, untranslated region.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.02.059

BMPs play an important role during development and regulate many processes, including cellular proliferation, adhesion, differentiation, inflammation, and apoptosis.[13] BMPs initiate signaling by binding cooperatively to transmembrane serine-threonine kinase receptors types 1 and 2, triggering the phosphorylation and activation of the type 1 receptor by the type 2 receptor kinase. The activated type 1 receptor phosphorylates SMADs 1, 5, and 8, and this permits their association with SMAD4. This heteromeric complex then translocates to the nucleus and regulates the transcription of genes specific for the BMP pathway.

In this study, we set out to investigate the expression of elements of the BMP pathway in CRC cell lines and patient specimens as a first step in determining whether the BMP pathway plays a role in sporadic CRC. Because TGFβR2 and ACVR2 losses are specific to MSI CRC,[2,8] we were also interested to see whether the expression of components of the BMP pathway was related to MSI status.

Materials and Methods

Cell Culture

CACO2, DLD1, SW480, LOVO, SW48, HT29, and HCT116 colon cancer cell lines were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (Gibco, Paisley, Scotland) with 4.5 g/L glucose and L-glutamine, penicillin (50 U/mL), streptomycin (50 μg/mL), and 10% fetal calf serum (Gibco).

Immunoblotting

Cells were scraped into sample buffer (125 mmol/L Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 2% β-mercaptoethanol, 20% glycerol, 1 mg bromophenol blue). Protein concentration was measured using the RC DC protein assay kit (Bio-Rad, Hercules, CA). The lysates were sonicated and then heated at 95°C for 5 minutes. A total of 50 μg of protein was loaded onto sodium dodecyl sulfate/polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blots were blocked in block buffer (2% low-fat milk powder in Tris-buffered saline with 1% Triton X-100 [TBST]) and incubated overnight at 4°C with primary antibody in TBST with 0.2% low-fat milk powder. Primary antibodies to BMPR1a (goat polyclonal), BMPR1b (mouse monoclonal), and BMPR2 (goat polyclonal) were from R&D Systems (Abingdon, England). SMAD4 (mouse monoclonal) and β-actin (rabbit polyclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were then incubated for 1 hour at room temperature in 1:2000 horseradish peroxidase–conjugated corresponding secondary antibody (Dako, Glostrup, Denmark) in block buffer. Finally, blots were incubated in Lumilite Plus (Boehringer Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer Mannheim).

Selection of Patient Material

An overview of the clinicopathologic data is available as Supplementary Table 1 (see supplemental material online at www.gastrojournal.org). Tissue from 72 CRC cases between 2002 and 2004 from the archives of the Pathology Department at the Academic Medical Centre, Amsterdam, was used for the compilation of the tissue microarray (TMA). The study was approved by the investigator's institutional review board.

Construction of the TMA

A Manual Tissue Arrayer MTA-1 (Beecher Instruments, Sun Prairie, WI) was used for the construction of the TMA. Three cores of tissue from each cancer specimen were used and, for each cancer case, one core from the corresponding normal colon.

Immunohistochemistry

TMA blocks were sectioned (4 μm), deparaffinized, immersed in 0.3% H_2O_2 in methanol for 20 minutes, and heat treated at 100°C (pH 9) for 10 minutes. Sections were blocked with 5% normal goat serum for 10 minutes followed by incubation for 1 hour with the primary antibody at room temperature. Rabbit polyclonal antibodies to BMPR2 were used at a concentration of 1:400. The specificity of the antibodies has been shown previously.[14] Mouse monoclonal antibodies to SMAD4 were from Santa Cruz Biotechnology (1:1600). The PowerVision Poly-HRP detection system (ImmunoVision Technologies, Daly City, CA) was used to visualize the antibody binding sites. Sections were counterstained with hematoxylin. Negative control sections for all antibodies were processed in an identical manner after omitting the primary antibody and showed no staining.

Immunohistochemistry for Phosphorylated SMAD 1, 5, and 8

As described above for general immunohistochemistry except that slides were boiled for 10 minutes in 0.01 mol/L sodium citrate, pH 6.0, and blocked with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% [vol/vol] Tween 20, pH 8.0) for 30 minutes. Slides were incubated with primary rabbit polyclonal antibodies to Phospho-Smad1/5/8, which recognizes the doubly phosphorylated forms of Smad1 (Ser463/465), Smad5 (Ser463/465), and Smad8 (Ser426/428) (Cell Signaling, Beverly, MA), at a concentration of 1:50 overnight at 4°C in phosphate-buffered saline with 0.1% Triton X-100 and 1% bovine serum albumin and then incubated with biotinylated secondary goat anti-rabbit antibodies (Dako) at a concentration of 1:200 at room temperature for 1 hour in phosphate-buffered saline with 10% human serum. Slides were then incubated

with horseradish peroxidase–conjugated ABComplex (Dako) for 1 hour, and peroxidase activity was detected with Fast DAB (Sigma Chemical Co, St Louis, MO).

Tissue Microdissection and DNA Extraction

Enriched tumor tissue (minimally 75% tumor cells) was microdissected from 4-μm formalin-fixed, paraffin-embedded tumor sections using a sterile needle, and DNA was extracted using the DNeasy Tissue Kit (Qiagen, Venlo, The Netherlands).

Microsatellite Analysis

MSI status was determined as previously described.[15] Analysis was performed using an automated ABI 377 or ABI 3100 sequencer (Applied Biosystems, Foster City, CA) with a GeneScan 350ROX size standard (Applied Biosystems) and the manufacturer's GeneScan 3.7 software. MSI tumors were defined as having $\geq$2 of 5 markers with novel alleles compared with matched normal DNA, whereas microsatellite stable (MSS) tumors had no novel alleles.

DNA Sequencing

DNA samples were amplified with specific primers in a reaction containing PCR Master Mix (Abgene, Rockford, IL) and 3 μmol/L of each primer. Polymerase chain reaction (PCR) was performed over 40 cycles of 94°C, 50°C, and 72°C of 1 minute each, preceded by a 5-minute denaturing step at 94°C and followed by a 10-minute extension step at 72°C. The correct product size was verified on 2% agarose gel. The PCR product was purified using a PCR purification kit (Qiagen) and was sequenced on an ABI 377 or 3100 genetic analyzer (Applied Biosystems) using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). BMPR2 was sequenced at the Leiden Genome Technology Center (www.lgtc.nl). Primers were designed with Primer3 software.[16] The sequences of all primers used are given in Supplementary Table 2 (see supplemental material onlineat www.gastrojournal.org).

RNA Isolation and Reverse-Transcription PCR

Total RNA was isolated using TRIzol (Invitrogen, Breda, The Netherlands). Complementary DNA was synthesized from 1 μg of total RNA using oligo dT primers and Superscript II MMLV reverse transcriptase (Life Technologies, Carlsbad, CA). PCR for BMPR2 was performed as in Emmanuele et al.[17]

Luciferase Reporter Assay

Cells were transiently transfected with either a BMPR2 plasmid or pmaxGFP control vector (Amaxa GmbH, Cologne, Germany) in combination with the BRE-Luc vector[18] and a cytomegalovirus (CMV) promoter-driven Renilla luciferase vector (Promega, Madison, WI) using Lipofectamine Plus (Invitrogen). After 24 hours of treatment with 100 ng/mL BMP2, luciferase activity was assayed using the Dual-Glo Luciferase Assay System (Promega) on a luminometer (Berthold Technologies, Bad Wildbad, Germany). Each firefly luciferase value was corrected for its cotransfected Renilla luciferase value.

Generation of pCMV-Luc-BMPR2 3′ Untranslated Region (wt/mut)

The CMV promoter was cut from the pcDNA3 vector with *Sal*I/*Hin*dIII and cloned into pGL3-basic cut with *Xho*I/*Hin*dIII using T4 ligase (New England Biolabs, Ipswich, MA). A ~9.4-kilobase fragment of the BMPR2 3′ untranslated region (UTR) was amplified from the genomic DNA of SW480 (BMPR2-wt) and DLD1 (BMPR2-mut) cells by nested PCR using Phusion High-Fidelity PCR Master Mix (Finnzymes) with introduction of *Nhe*I recognition sites on 5′ and 3′ ends of the product. The product was digested with *Nhe*I and cloned into pCMV-Luc digested with *Xba*I. The constructed plasmids were confirmed by direct sequencing and used for luciferase assays.

Statistical Analysis

Statistical analysis was performed using SPSS version 11.5 for Windows (SPSS Inc, Chicago, IL). The χ^2 test and Fisher exact test were used as appropriate. $P < .05$ was considered statistically significant.

Two-way hierarchical cluster analysis was performed using JMP 6.0.0. (SAS Institute, Inc, Cary, NC). Only first- and second-generation clusters were used for classification purposes.

TMA Analysis, GeneScan Analysis, and Methylation Analysis

See supplementary methods (see supplemental material online at www.gastrojournal.org).

Results

Expression of the Human BMP Receptors and SMAD4 in CRC Cell Lines

Seven CRC cell lines were investigated for the expression of BMP receptors and SMAD4 at protein level by immunoblotting (Figure 1*A*). BMPR1a is expressed at the protein level in 6 CRC cell lines and absent in DLD1. BMPR1b is expressed in all cell lines tested. BMPR2 protein expression, however, is reduced or absent in MSI HCT116, DLD1, SW48, and LOVO cells while its expression is normal in MSS SW480, HT29, and CACO2 cells. Interestingly, the expression of SMAD4 shows a completely reverse pattern, being negative in all MSS and positive in all MSI cell lines, as shown previously.[19]

Messenger RNA (mRNA) levels of BMPR2 as judged by semiquantitative reverse-transcription PCR performed in the same cell lines correlate well with the protein levels found by Western blot with only one exception. SW48

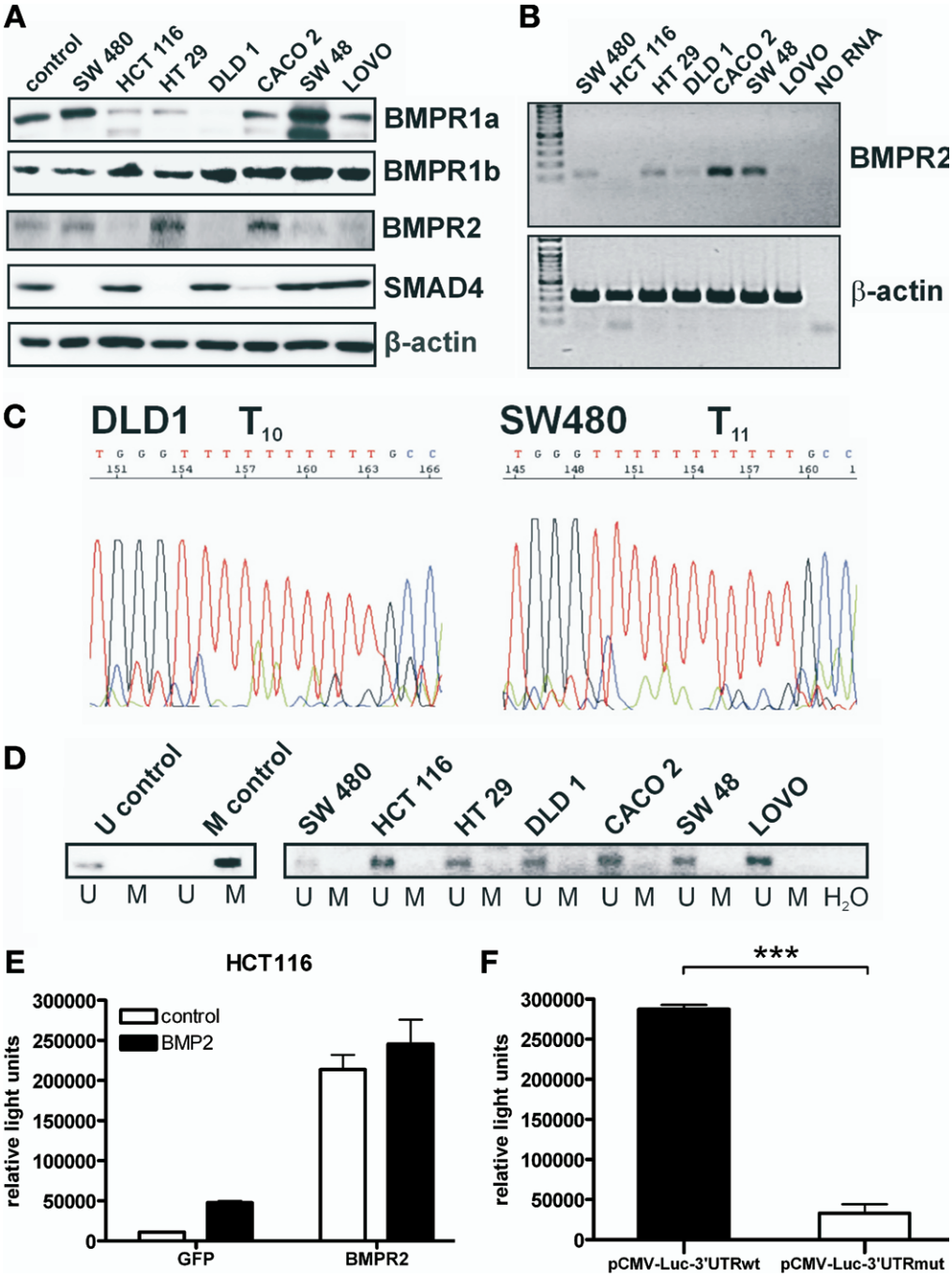


Figure 1. (*A*) Immunoblot for BMPR1a, BMPR1b, BMPR2, and SMAD4 in 7 colon cancer cell lines. A total of 50 μg of protein from cell lysates was loaded per lane and analyzed by blotting with the corresponding specific antibody. SAOS-2 osteosarcoma cells were used as a positive control. Equal loading was confirmed by showing equal β-actin levels. (*B*) DNA sequence analysis of the A_{11} BMPR2 microsatellite tract in CRC cell lines. MSI DLD1 cells show a 1–base pair contraction to T_{10} in the reverse strand, while the MSS SW480 cell line shows wild-type T_{11} in the reverse strand. (*C*) Reverse-transcription PCR for BMPR2 in 7 colon cancer cell lines. A single product of the expected size (115 base pairs) was seen as shown previously. β-actin was used as a control. (*D*) Methylation-specific PCR analysis of the CpG island in the BMPR2 5′ region in colon cancer cell lines. PCR products specific for unmethylated (U) and methylated (M) CpG sites are shown. U control, human genomic DNA from peripheral blood lymphocytes; M control, human genomic DNA treated in vitro with *Sss*I methyltransferase. (*E*) Functional analysis of the consequences of BMPR2 loss. HCT116 cells were transfected with either BMPR2 or a GFP control vector, and the activity of the BMP pathway was determined using the BRE-Luc reporter construct. After 24 hours of treatment with 100 ng/mL BMP2, luciferase activity was assayed. GFP transfected cells show slight up-regulation of BRE-luc activity after BMP2 treatment. Transfection with BMPR2 increases BRE-luc activity 20-fold. This high activity increases only modestly with BMP2 treatment. (*F*) The influence of mutations in the BMPR2 3′UTR on protein expression. HCT116 cells were transfected with equal amounts of pCMV-Luc-3′UTR wt or mut vectors containing the full-length BMPR2 3′UTR from SW480 and DLD1 cell lines, respectively, in combination with a control CMV promoter-driven Renilla luciferase vector, and a dual luciferase assay was performed. The mutated 3′UTR leads to a 10-fold reduction in luciferase expression compared with wt 3′UTR (***$P < .0001$).

Table 1. BMPR2, ACVR2, and TGFβR2 Mutations in Colon Cancer Cell Lines and in MMR-Deficient Colon Cancer Specimens With Altered BMPR2 Protein Expression

Cell line	MSI status	BMPR2 A_{11}	ACVR2 A_8	TGFβR2 A_{10}
SW480	Negative	A_{11}	A_8	A_{10}
HT29	Negative	A_{11}	A_8	A_{10}
CACO 2	Negative	A_{11}	A_8	A_{10}
HCT116	Positive	A_{10}	A_7	A_9
DLD1	Positive	A_{10}	A_7	A_9
SW48	Positive	A_{10}	A_7	A_9
LOVO	Positive	A_8	A_7	A_9
Patient no.				
1	Positive	A_9	A_8	A_{10}
2	Positive	A_9	A_8	A_9
3	Positive	A_9	A_7	A_9
4	Positive	A_{10}	A_7	A_9
5	Positive	A_{10}	A_7	A_9
6	Positive	A_{10}	A_7	A_9
7	Positive	A_{10}	[a]	A_{10}
8	Positive	A_{10}	A_8	A_{10}
9	Positive	A_{10}	A_7	A_{10}
10	Positive	A_{11}	A_7	A_{10}
11	Positive	A_{11}	A_8	A_{10}

[a]Insufficient DNA.

cells show attenuated levels of BMPR2 protein but normal levels of mRNA (Figure 1*B*).

Because there is a clear association between altered BMPR2 expression and microsatellite instability, we checked the mRNA sequence of BMPR2 for the presence of microsatellites. We found a 7 adenine tract in exon 12 and a long 11 adenine tract in the 3′UTR. We first sequenced the coding A_7 microsatellite and found a monoallelic frameshift mutation in 2 MSI cell lines, HCT116 and LOVO (1742delA), leading to a stop codon. GeneScan analysis confirmed the heterozygous nature of this mutation (Supplementary Figure 1; see supplemental material online at www.gastrojournal.org). We then went on to check for this mutation in MSI-high cancer specimens with attenuated BMPR2 expression (n = 11) by direct sequencing. None of the 11 human cancer specimens contained this mutation (data not shown). We then sequenced the A_{11} in the 3′UTR as well as the A_{10} of TGFβR2 and A_8 of ACVR2 (Table 1), both coding microsatellites described by previous investigators.[2,8] The results show that BMPR2 is mutated at A_{11} in all MSI cell lines tested and in none of the MSS cell lines (Figure 1*C* and Table 1). All of our MSI cell lines also show frameshift mutations within A_{10} of TGFβR2 and A_8 of ACVR2.

To exclude other possible mutations explaining BMPR2 loss, we sequenced the whole coding sequence of BMPR2 in our 7 cell lines. Apart from the heterozygous 1742delA mutations in HCT116 and LOVO found previously, we found a monoallelic point mutation in exon 6 (631C>T) leading to a stop codon in DLD-1 (data not shown).

An alternative explanation for BMPR2 protein and mRNA loss could be promoter hypermethylation leading to gene silencing in MSI cancers with a CpG island methylator phenotype and consequent loss of MLH1 expression. This mechanism would not, however, explain the loss of BMPR2 expression we see in tumors and cell lines with loss of MSH2 expression. We performed both methylation-specific PCR and bisulfite sequencing for the BMPR2 promoter CpG island region. All 7 CRC cell lines contain only unmethylated alleles of BMPR2 (Figure 1*D*). Bisulfite sequencing in the 7 cell lines showed no methylation of the promoter CpG island within the examined region.

Reconstitution of BMPR2-Negative Cells With BMPR2 Leads to Activation of the BMP Pathway

To investigate the functional consequences of BMPR2 loss, we used a reporter of BMP pathway activity and a wt-BMPR2 plasmid. BMPR2-negative HCT116 cells were cotransfected with either the BMP pathway activity reporter construct (BRE-Luc) and green fluorescent protein (GFP) or with BRE-Luc and wt-BMPR2. Reconstitution of HCT116 cells by transient transfection with wt-BMPR2 induces 20-fold activation of BMP pathway activity (Figure 1*E*) even in the absence of exogenous BMP. The addition of exogenous BMP2 leads to only a modest further increase in BMP pathway activity.

Reduced Expression of Luciferase When Coupled to the Mutant BMPR2 3′UTR (mut) Compared With the Wild-Type 3′UTR (wt)

To determine the functional significance of the mutation in the 3′UTR of BMPR2, we generated pCMV-Luc-BMPR2 3′UTR wt and mut vectors containing the full-length BMPR2 3′UTR from SW480 and DLD1 cell lines, respectively. HCT116 cells were then transfected with equal amounts of the wt or mut construct together with the control construct, and the dual luciferase reporter assay was performed 24 hours later. The mutated 3′UTR resulted in 10-fold reduction of luciferase expression compared with wt 3′UTR ($P < .0001$) (Figure 1*F*).

Expression of the BMP Receptors and Signal Transduction Elements in Human Sporadic CRCs

To further investigate the role of the BMP pathway in CRC, we constructed a TMA. The clinical characteristics of the study population are listed in Supplementary Table 1. Based on the absence of hMLH1 and hMSH2, as judged by immunohistochemistry, 13 patients (18.1%) were classified as mismatch repair (MMR) deficient and 59 (81.9%) as MMR proficient.

We performed immunohistochemical staining for BMPR1a, BMPR1b, BMPR2, and SMAD4 (Figure 2*A*). MMR-deficient CRCs show frequent loss of expression of

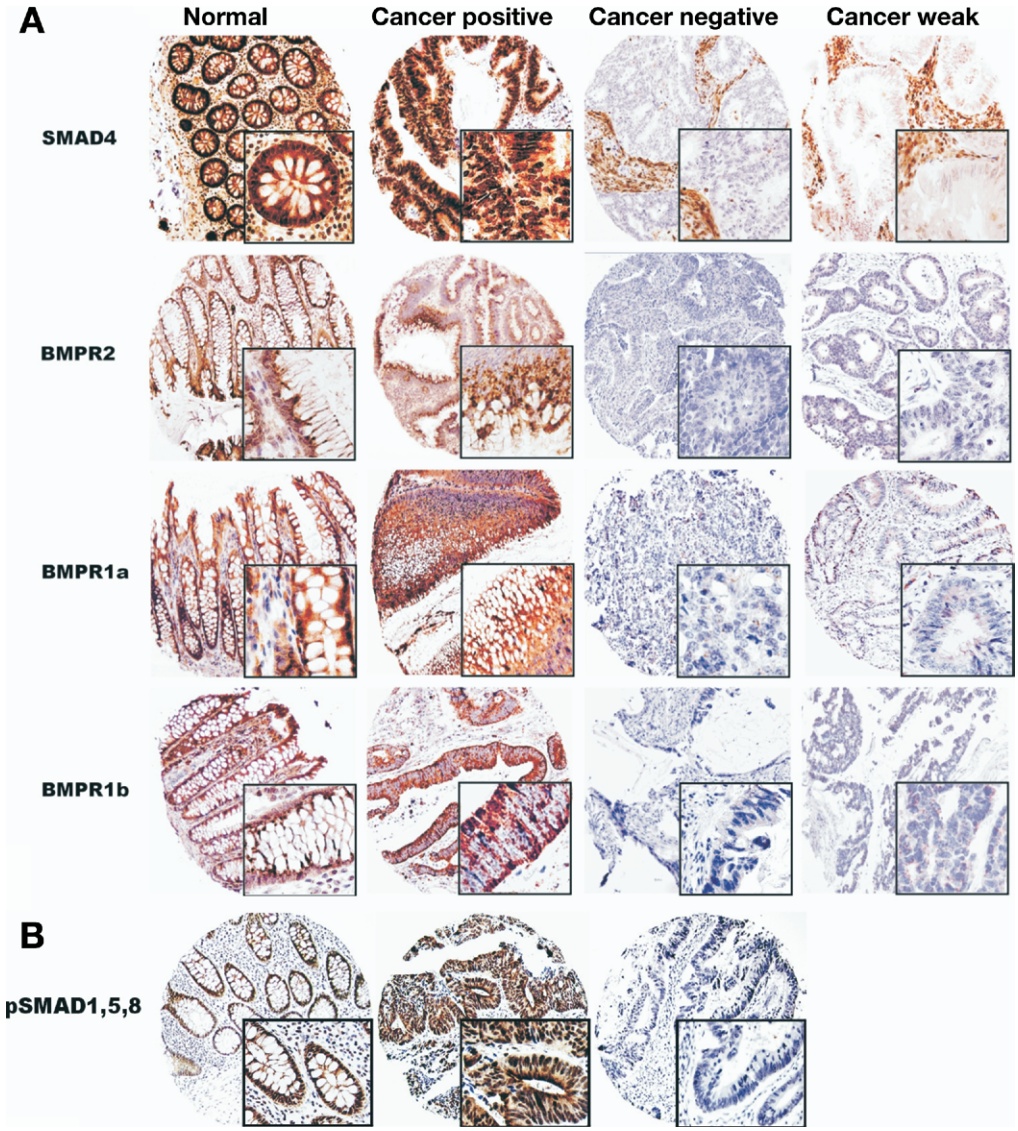


Figure 2. (*A*) Immunohistochemistry for SMAD4, BMPR2, BMPR1a, and BMPR1b in normal human colon and colon tumors. Representative cores from normal and cancer specimens to show positive, negative, or weak expression. (*B*) Immunohistochemistry for pSMAD1/5/8 in normal human colon and colon tumors. Representative cores from normal and cancer specimens to show positive and negative expression of pSMAD1/5/8.

BMPR2 ($P < .0001$) compared with MMR-proficient cancers (Table 2), in agreement with our immunoblot results. The frequency of the loss of BMPR1a and BMPR1b expression does not differ significantly between these 2 groups of cancers.

To investigate the influence of the abnormal expression of BMPRs and SMAD4 on the activity of the BMP pathway in sporadic colon cancers, we performed immunohistochemical staining for pSMAD1/5/8 (Figure 2*B*) and found a highly statistically significant association between defective expression of the BMP pathway components and negative nuclear staining for pSMAD1/5/8 ($P < .0001$) (Table 3).

Interestingly, we observed a mutually exclusive pattern of loss of expression of BMPR2 and SMAD4 in sporadic CRC (Table 4 and Figure 3*A*). None of the cancers show negative expression of both BMPR2 and SMAD4. This is the same expression we observed in cell lines. Only 4% (3) of cancers have weak staining for both, and 33% (24) cancers are positive for both. All other cancers exhibit either BMPR2 or SMAD4 loss. To clearly show the selective loss of BMPR2 expression in tumor tissue, we performed immunohistochemistry on sections containing normal and cancer tissue on one slide (Figure 3*B*). Here it can be seen that loss of BMPR2 staining occurs specifically in the tumor.

To look for further correlations, we performed hierarchical cluster analysis of the TMA data. This results in 4 clusters of carcinomas (based on the first- and second-level branches of the tree) (Figure 3*C*). Carcinomas in cluster C1 are the only carcinomas with normal BMP pathway activity as assessed by pSMAD1/5/8. They express all components of the BMP pathway and the MMR proteins normally. Carcinomas in cluster C2 are MMR-deficient cancers with aberrant BMPR2 and positive SMAD4 expression as main characteristics. In contrast, cluster C3 consists of MMR-proficient cancers with aberrant SMAD4 and positive BMPR expression. Cancers in

Table 2. Expression of BMPRs and SMAD4 in CRC Specimens

Characteristic, n (%)	All cases (n = 72)	MMR proficient (n = 59)	MMR deficient (n = 13)	*P* value
BMPR1a				
0 (negative)	2 (2.8)	1 (1.7)	1 (7.7)	.082
1 (weak)	15 (20.8)	10 (16.1)	5 (38.5)	
2 (positive)	55 (76.4)	48 (83.9)	7 (53.8)	
BMPR1b				
0 (negative)	4 (5.6)	3 (5.2)	1 (7.7)	.148
1 (weak)	7 (9.9)	4 (6.9)	3 (23.1)	
2 (positive)	60 (84.5)	51 (87.9)	9 (69.2)	
BMPR2				
0 (negative)	9 (12.5)	4 (6.8)	5 (38.5)	<.0001
1 (weak)	19 (26.4)	13 (22.0)	6 (46.2)	
2 (positive)	44 (61.1)	42 (71.2)	2 (15.4)	
SMAD4				
0 (negative)	7 (9.7)	7 (11.9)	0 (0.0)	.41
1 (weak)	19 (26.4)	16 (27.1)	3 (23.1)	
2 (positive)	46 (63.9)	36 (61.0)	10 (76.9)	

NOTE. Percentages in parentheses refer to the percentage of cases in each individual column. For example, BMPR1a staining was positive in 76.4% of all cancers, 83.9% of MMR-proficient cancers, and 53.8% of MMR-deficient cancers. Statistical analysis was performed using the Fisher exact test for a significant difference between MMR-proficient and MMR-deficient cancers over the table of 6 values obtained for each characteristic.

cluster C4 are also MMR proficient but with negative BMPR2 and high numbers of BMPR1a negative expression. Clusters 2, 3, and 4 all show attenuated BMP pathway activity with negative nuclear pSMAD1/5/8 expression.

Association Between BMPR2 Altered Protein Expression and A_{11} Tract Mutations of BMPR2 in MSI Colon Cancers

We selected all (n = 11) tumors with attenuated expression of BMPR2 and loss of MLH-1 or MSH-2 expression and first determined MSI status in these samples. All 11 tumors were MSI high (data not shown). We then sequenced A_{11} of BMPR2 (Figure 4), A_{10} of TGFβR2, and A_8 of ACVR2. Nine out of 11 colon carcinomas analyzed show deletions of 1 or 2 adenines from the A_{11} of BMPR2 (Table 1). In contrast, none of the MMR-proficient tumors with positive expression of BMPR2 used as control (n = 8) show this deletion. We also analyzed the same samples for mutations within A_{10} of TGFβR2 and A_8 of ACVR2. Five out of 11 colon carcinomas had a one adenine deletion in the polyadenine tract of TGFβR2 and 6 out of 10 carcinomas had mutations in A_8 of ACVR2. None of the control tumors were mutated. Our results show a very tight correlation between attenuation of BMPR2 expression in MSI cancers and mutations in A_{11} of BMPR2.

Table 3. Association Between Nuclear Localization of pSMAD1/5/8 and Expression of the Components of the BMP Pathway

	pSMAD1/5/8		
	Negative	Positive	*P* value
BMP pathway defective[a]	44 (61.1%)	6 (8.3%)	<.0001
Normal	6 (8.3%)	16 (22.2%)	

NOTE. n = 72. Percentages in parentheses refer to the percentage of the total number of cases.
[a]Defective means negative or weak score in any one staining (BMPR1a, BMPR1b, BMPR2, or SMAD4).

Table 4. Mutually Exclusive Nature of BMPR2 and SMAD4 Loss

	BMPR2		
	Negative	Weak	Positive
SMAD4			
Negative	0 (0.0%)[a]	0 (0.0%)[a]	7 (9.7%)[b]
Weak	3 (4.2%)[a]	3 (4.2%)[a]	13 (18.1%)[b]
Positive	6 (8.3%)[b]	16 (22.2%)[b]	24 (33.3%)[c]

NOTE. n = 72. Percentages in parentheses refer to the percentage of the total number of cases.
[a]Negative/weak for both.
[b]Mutually exclusive.
[c]Positive for both.

Discussion

While it is clear that alterations in the BMP pathway lead to inherited forms of CRC, whether the BMP pathway is involved in sporadic CRC has received little attention. Here we show frequent inactivation of the BMP pathway in sporadic colon cancers associated with loss of BMPR2 and SMAD4 expression. Our results show that expression of BMPR2 is impaired in MSI colon cancer cell lines and colon cancer specimens. In contrast, all MSS cell lines express BMPR2 but do not express SMAD4, a key molecule for TGF-β, activin, and BMP pathways.

These findings contrast with those of Beck et al,[20] who investigated the expression of BMP pathway components in 2 cell lines and 13 primary cancer specimens and concluded that the BMP pathway was intact. However, they did not investigate BMPR2 and did not specifically determine nuclear localization of pSMAD1/5/8 in their tumor specimens. This might explain the discrepancy between our findings and theirs.

Our finding that cell lines with microsatellite instability and the previously described TGFβR2 A_{10} and ACVR2 A_8 mutations also express abnormal levels of BMPR2 is interesting in that it may indicate that the MSI cell lines tested have multiple defects in their ability to respond to growth inhibitory stimuli of TGF-β superfamily ligands. Evidence is accumulating that there is some redundancy at the receptor 2 level in TGF-β family signaling. Despite homozygous mutations of the TGFβR2, some colon cancer cell lines (LOVO and SW48) respond to TGF-β 1,

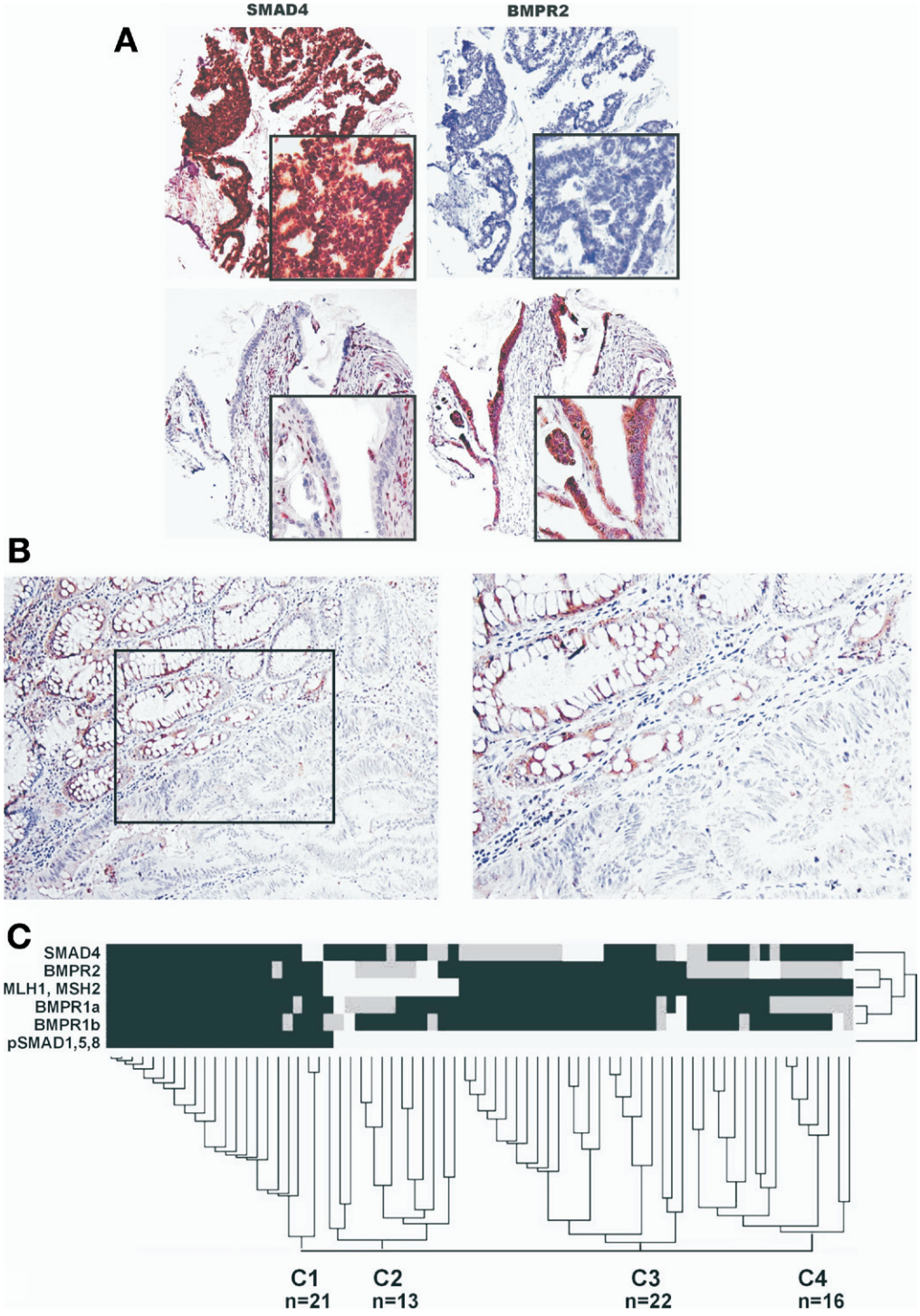


Figure 3. (*A*) Immunohistochemistry for SMAD4 and BMPR2 showing the mutually exclusive nature of SMAD4 and BMPR2 expression in colon cancer specimens. The same tumor stained for SMAD4 or BMPR2 is shown in each case. Note that the tumor with positive expression of SMAD4 has negative BMPR2 protein expression and the tumor with negative SMAD4 staining strongly expresses BMPR2 protein. (*B*) Selective loss of expression of BMPR2 in an MSI tumor. Note the expression of BMPR2 protein in normal colonic epithelium and loss of expression in the tumor. Stromal tissue is negative in both normal and carcinoma tissue. (*C*) Heat map and dendrograms to show the results of hierarchical cluster analysis of the expression of MMR proteins, BMPRs, SMAD4, and pSMAD1/5/8 in 72 CRC specimens. *Rows* represent protein expression and *columns* represent individual tumors. *Black cells* represent positive expression, *gray cells* represent weak expression, and *white cells* represent negative expression.

showing growth inhibition.[21] Interestingly, these are cell lines that show low but some level of BMPR2 in our immunoblots. Others have shown TGF-β–specific gene activation on overexpression of ACVR2 in TGFβR2 and ACVR2 mutated colon cancer cell lines.[22] Similarly, BMPs can signal via ACVR2, have overlapping binding specificities with activins, and share some of the functional effects of activins[23,24] despite the fact that earlier binding studies indicated ligand receptor 2 interaction specificity.[25] This would suggest that loss of one of the 3 type 2 receptors is insufficient for complete pathway disruption and that simultaneous loss of several of these receptors is required.

Heterozygous mutations in BMPR2 are associated with human familial and idiopathic pulmonary arterial hypertension, and reduced levels of expression of BMPR2 protein have been found in the lungs of all patients with pulmonary hypertension examined.[26–28] The human BMPR2 gene con-

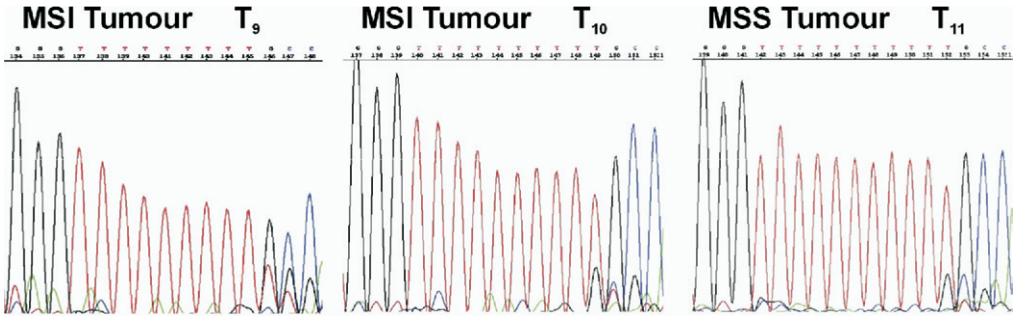


Figure 4. Representative examples of DNA sequence analysis of the A11 BMPR2 microsatellite tract in MSI and MSS tumors. The MSI tumors show a 1– or 2–base pair contraction to T10 or T9 in the reverse strand, while the MSS tumor shows wild-type T11 in the reverse strand.

tains 7 kilobases of 3′UTR sequence, which is believed to contribute to posttranscriptional regulation of mRNA turnover. It has been suggested that the reduced pulmonary endothelial expression of BMPR2 found in patients without the classic mutations in the coding regions might arise from posttranscriptional down-regulation of BMPR2 RNA, and it has been shown that reduced BMPR2 expression could be enhanced by medicines increasing the posttranscriptional stability of BMPR2 mRNA.[29] Our finding that the long polyadenine tract in the 3′UTR of BMPR2 mRNA is mutated with high frequency in MMR-deficient cancers and coupled with altered BMPR2 expression might indicate a causative link. This is supported by 3 lines of evidence. Firstly, there is conservation of microsatellites in the 3′UTR of genes and indirect evidence that mutations in these are not merely bystander events.[30] Secondly, the expression of several other proteins has been shown to be altered by mutations at microsatellites in the 3′UTR such as CEACAM1[31] and CDK2-AP1.[32] These mutations have been shown to affect RNA stability. Finally, we show that a mutant form of the BMPR2 3′UTR coupled to a luciferase construct leads to a 10-fold reduction in luciferase protein expression compared with the wild-type BMPR2 3′UTR sequence. Our reverse-transcription PCR data show reduced mRNA levels of BMPR2 in essentially the same pattern seen at protein level. This suggests that protein levels are being influenced at the mRNA level by influencing mRNA stability. While our functional analysis of the effect of the BMPR2 3′UTR mutations confirms their importance, exactly how they do this is a subject for further study.

We have excluded alternative explanations for BMPR2 loss such as coding sequence mutations and promoter methylation as far as possible. This is in agreement with the results of screens in CRC for mutations in gene coding sequences[33] and for genes silenced by methylation,[34] which have not identified BMPR2. We do find heterozygous coding mutations in several MSI cell lines, but this is an unlikely explanation of loss of BMPR2 protein expression especially because we do not find this in patient specimens. However, in primary pulmonary hypertension, heterozygous mutations are believed to be the cause of the abrogated BMPR2 expression seen.[27]

Loss of heterozygosity due to chromosomal instability is not seen in MSI tumors, making large deletions an unlikely explanation for BMPR2 loss in this tumor type.[35] Furthermore, we find a heterozygous pattern in the 3′UTR BMPR2 sequence in most of the cell lines, suggesting the presence of 2 alleles, and studies of chromosomal instability in CRC have not revealed loss of heterozygosity in chromosome 2.[36]

We explored whether changes in the protein expression of BMPR2 or SMAD4 influence the activity of the BMP pathway as judged by pSMAD1/5/8 nuclear staining. According to our data, 70% of sporadic colon carcinomas exhibit negative pSMAD1/5/8 nuclear staining. With the corroborating evidence provided by the SMAD4 and BMPR staining, it seems likely that this indicates an inactive BMP pathway in these tumors.

To confirm the functional importance of BMPR2 loss, we reconstituted HCT116 cells by reintroducing wild-type BMPR2. This induces a 20-fold increase in BRE-Luc reporter construct activity, indicating strong activation of the BMP pathway even without the addition of exogenous BMP. Similar results have previously been obtained when the SW480 (SMAD4-null) cell line was reconstituted with wt-SMAD4.[20]

In summary, our data show extensive loss of BMP signaling in sporadic colon cancer. Loss of BMPR2 expression is associated with functionally important microsatellite mutations in the 3′UTR region. The loss of BMPR2 expression in MSI cancer occurs in combination with the known defects in TGFβR2 and ACVR2 expression. This would support the hypothesis that while a mutation in one of the SMADs is sufficient to cause neoplasia, mutations at the TGF-β superfamily receptor 2 level must occur in combination.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.02.059.

References

1. de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor-beta signaling in cancer. J Natl Cancer Inst 2000; 92:1388–1402.

2. Markowitz S, Wang J, Myeroff L, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 1995;268:1336–1338.
3. Biswas S, Chytil A, Washington K, et al. Transforming growth factor beta receptor type II inactivation promotes the establishment and progression of colon cancer. Cancer Res 2004;64: 4687–4692.
4. Takaku K, Miyoshi H, Matsunaga A, et al. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. Cancer Res 1999;59: 6113–6117.
5. Zhu Y, Richardson JA, Parada LF, et al. Smad3 mutant mice develop metastatic colorectal cancer. Cell 1998;94:703–714.
6. Thiagalingam S, Lengauer C, Leach FS, et al. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nat Genet 1996;13:343–346.
7. Jung B, Doctolero RT, Tajima A, et al. Loss of activin receptor type 2 protein expression in microsatellite unstable colon cancers. Gastroenterology 2004;126:654–659.
8. Howe JR, Roth S, Ringold JC, et al. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science 1998;280:1086–1088.
9. Howe JR, Bair JL, Sayed MG, et al. Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. Nat Genet 2001;28:184–187.
10. Haramis A-PG, Begthel H, van den Born M, et al. De novo crypt formation and juvenile polyposis on BMP Inhibition in mouse intestine. Science 2004;303:1684–1686.
11. Hardwick JC, Van Den Brink GR, Bleuming SA, et al. Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. Gastroenterology 2004;126:111–121.
12. Beppu H, Mwizerwa ON, Beppu Y, et al. Stromal inactivation of BMPRII leads to colorectal epithelial overgrowth and polyp formation. Oncogene 2008;27:1063–1070.
13. Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer 2003;3: 807–821.
14. Rosenzweig BL, Imamura T, Okadome T, et al. Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A 1995;92:7632–7636.
15. Loukola A, Eklin K, Laiho P, et al. Microsatellite marker analysis in screening for hereditary nonpolyposis colorectal cancer (HNPCC). Cancer Res 2001;61:4545–4549.
16. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–386.
17. Emmanuele L, Ortmann J, Doerflinger T, et al. Lovastatin stimulates human vascular smooth muscle cell expression of bone morphogenetic protein-2, a potent inhibitor of low-density lipoprotein-stimulated cell growth. Biochem Biophys Res Commun 2003;302:67–72.
18. Logeart-Avramoglou D, Bourguignon M, Oudina K, et al. An assay for the determination of biologically active bone morphogenetic proteins using cells transfected with an inhibitor of differentiation promoter-luciferase construct. Anal Biochem 2006;349:78–86.
19. Woodford-Richens KL, Rowan AJ, Gorman P, et al. SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. Proc Natl Acad Sci U S A 2001;98:9719–9723.
20. Beck SE, Jung BH, Fiorino A, et al. Bone morphogenetic protein signaling and growth suppression in colon cancer. Am J Physiol Gastrointest Liver Physiol 2006;291:G135–G145.
21. Ilyas M, Efstathiou JA, Straub J, et al. Transforming growth factor beta stimulation of colorectal cancer cell lines: type II receptor bypass and changes in adhesion molecule expression. Proc Natl Acad Sci U S A 1999;96:3087–3091.
22. Deacu E, Mori Y, Sato F, et al. Activin type II receptor restoration in ACVR2-deficient colon cancer cells induces transforming growth factor-β response pathway genes. Cancer Res 2004;64: 7690–7696.
23. Yamashita H, ten Dijke P, Huylebroeck D, et al. Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. J Cell Biol 1995;130:217–226.
24. Yu PB, Beppu H, Kawai N, et al. Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. J Biol Chem 2005;280:24443–24450.
25. Massague J, Like B. Cellular receptors for type beta transforming growth factor. Ligand binding and affinity labeling in human and rodent cell lines. J Biol Chem 1985;260:2636–2645.
26. Deng Z, Morse JH, Slager SL, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 2000; 67:737–744.
27. Lane KB, Machado RD, Pauciulo MW, et al. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. Nat Genet 2000;26:81–84.
28. Atkinson C, Stewart S, Upton PD, et al. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation 2002;105:1672–1678.
29. Hu H, Sung A, Zhao G, et al. Simvastatin enhances bone morphogenetic protein receptor type II expression. Biochem Biophys Res Commun 2006;339:59–64.
30. Suraweera N, Iacopetta B, Duval A, et al. Conservation of mononucleotide repeats within 3' and 5' untranslated regions and their instability in MSI-H colorectal cancer. Oncogene 2001;20: 7472–7477.
31. Ruggiero T, Olivero M, Follenzi A, et al. Deletion in a (T)8 microsatellite abrogates expression regulation by 3'-UTR. Nucleic Acids Res 2003;31:6561–6569.
32. Shin J, Yuan Z, Fordyce K, et al. A del T poly T (8) mutation in the 3' untranslated region (UTR) of the CDK2-AP1 gene is functionally significant causing decreased mRNA stability resulting in decreased CDK2-AP1 expression in human microsatellite unstable (MSI) colorectal cancer (CRC). Surgery 2007;142:222–227.
33. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. Science 2006;314:268–274.
34. Suzuki H, Gabrielson E, Chen W, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 2002;31:141–149.
35. Remvikos Y, Vogt N, Muleris M, et al. DNA-repeat instability is associated with colorectal cancers presenting minimal chromosome rearrangements. Genes Chromosomes Cancer 1995;12: 272–276.
36. Fensterer H, Radlwimmer B, Strater J, et al. Matrix-comparative genomic hybridization from multicenter formalin-fixed paraffin-embedded colorectal cancer tissue blocks. BMC Cancer 2007;7:58.

Received September 18, 2006. Accepted January 31, 2008.

Address requests for reprints to: James C. H. Hardwick, MD, PhD, Center for Experimental and Molecular Medicine, Room G2-105, Academic Medical Center, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands. e-mail: j.c.h.hardwick@lumc.nl; fax: (31) 20-6977192.

Supported by an EC FP6 grant for tumor host genomics (to E.W.) and the Dutch Cancer Society (L.L.K.).

The authors report that there is no conflict of interest to disclose.

The authors thank Maarten van Dinther for his assistance with the generation of constructs.

Supplementary Methods

TMA Analysis

Analysis was performed by 2 investigators independently in a blinded fashion. Expression was graded from 0 to 2 for BMPR1a, BMPR1b, and BMPR2 (0, no staining; 1, weak membrane or intracytoplasmic staining in less than 10% of the cells; 2, moderate to strong membrane or intracytoplasmic staining in more than 10% of the cells).

SMAD4 staining was scored as follows: 0, no staining, 1, weak nuclear staining or negative nuclear and weak intracytoplasmic staining in less then 10% of the cells; 2, moderate to strong nuclear staining in more than 10% of the cells. For MLH1 and MSH2 expression, samples with no nuclear staining in tumor cells were classified as negative and as positive if more than 10% of cells had nuclear staining intensity greater than that of negative control slides. Expression of pSMAD1/5/8 was graded as negative if less then 30% of cells showed weak nuclear staining or less than 10% of cells showed strong nuclear staining.

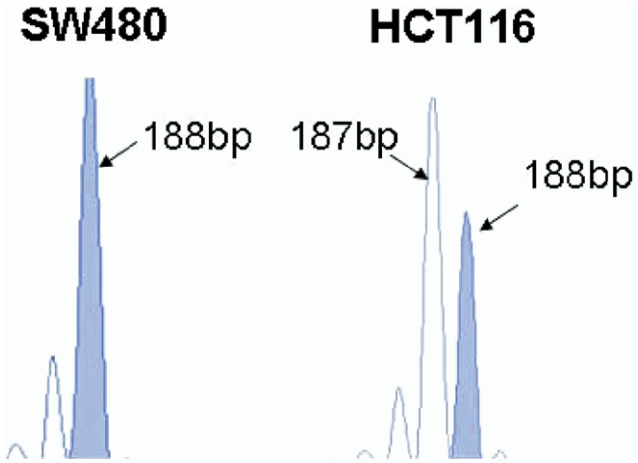
Methylation Analysis

Bisulfite treatment was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA). Bisulfite sequencing and methylation-specific PCR were performed as described previously.[1] The region within the BMPR2 promoter CpG island examined by bisulfite sequencing contains 52 CpG dinucleotides and lies between −1051 and −497 base pairs upstream of the predicted transcriptional start site.[2] Methylation-specific PCR examined a region between −389 and −272 base pairs. Primers were designed with MethPrimer software[3] and were designed to analyze the most proximal BMPR2 promoter region as predicted by Proscan[4] and previous functional analysis of the BMPR2 promoter.[2] This region also falls within a CpG island (criteria used: island size >400, GC % >50.0, obs/exp >0.6). This is a region where methylation correlates with transcriptional repression in other genes as reviewed by Ushijima.[5] Human genomic DNA from peripheral blood lymphocytes was used as the unmethylated control. Human genomic DNA treated in vitro with *Sss*I methyltransferase (New England Biolabs, Beverly, MA) was used as positive control for the methylated reaction.

For the GeneScan analysis, PCR was performed with specific primers as shown in Supplementary Table 1. The forward primer was HEX labeled. The GeneScan-500 ROX internal-lane size standard (Applied Biosystems) was added to the PCR product, the mix was analyzed on an ABI Prism 3100 genetic analyzer, and the size of the DNA fragments containing the 7A microsatellite was measured using GeneScan 3.1 analysis software.

Supplementary References
Additional References

1. Herman JG, Graff JR, Myohanen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–9826.
2. Hu H, Sung A, Zhao G, et al. Simvastatin enhances bone morphogenetic protein receptor type II expression. Biochem Biophys Res Commun 2006;339:59–64.
3. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002;18:1427–1431.
4. Prestridge DS. Predicting Pol II promoter sequences using transcription factor binding sites. J Mol Biol 1995;249:923–932.
5. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 2005;5:223–231.



Supplementary Figure 1. GeneScan analysis of the 188–base pair DNA fragment containing the 7A microsatellite. SW480 cells have only the wild-type 7A tract on sequencing and all fragments are 188 base pairs in length, while HCT116 cells with a heterozygous mutation in the 7A tract by sequencing show fragments of both 187 base pairs and 188 base pairs in length, confirming the heterozygous nature of this mutation.

Supplementary Table 1. Characteristics of Patients and Tumors

Characteristic	All cases	MMR-proficient CRC	MMR-deficient CRC	*P*
Patients, n (%)	72 (100)	59 (81.9)	13 (18.1)	
Age at presentation, y				
Mean (±SD)	69.85 (11.8)	68.8 (12.2)	73.7 (9.7)	.62
Median	70	68	73	
Range	30–92	30–92	51–88	
Sex, n. (%)				
Male	37 (51.4)	27 (45.8)	10 (68.8)	.065
Female	35 (48.6)	32 (54.2)	3 (23,1)	
Site of tumor, n (%)				
Colon	45 (62.5)	35 (59.3)	10 (76.9)	.35
Rectum	27 (37.5)	24 (40.7)	3 (23.1)	
Tumor grade, n (%)				
Well differentiated	9 (12.5)	8 (13.6)	1 (7.6)	.24
Moderately differentiated	44 (61.1)	38 (64.4)	6 (46.2)	
Poorly differentiated	19 (26.4)	13 (22.0)	6 (46.2)	

Supplementary Table 2. Primers

Target	Forward Primer 5′- 3′	Reverse Primer 5′- 3′
BMPR2 11A	ATTAGGTCACTGAAAGAACT	GCATATTACTTAGCTTCTCT
BMPR2 7A	TCCATCATACTGACAGCATCG	TGTGGTGTTTGTGGTTGTTG
TGFβR2 10A	TTCTCTCTCTCCCTCTCCCC	TGCACTCATCAGAGCTACAGG
ACVR2 9A	GTTGCCATTTGAGGAGGAAA	CCTCTGAAAAGTGTTTTATTGGAA
BMPR2 RT-PCR	GATGGCAAATCAGGATCAGG	CCTCACAGTCCAGCAATTCAG
BMPR2 MSP U	TTAGGAGTTTAGAGTTGTGGGAGAAT	CTCCCATCAATAACTCCTATAAACAA
BMPR2 MSP M	GTTTAGAGTTGCGGGAGAAC	CGTCAATAACTCCTATAAACGAA
BMPR2 Bis.seq.	AATAATAGAGGGTAGTTTTGTTTTT	AAAAACACTTCCAATAACTCC
pCMV-Luc-3′UTR		
~9.4kb fragment	AAAAATATTGTATGCCAGGTGC	AAAGCAAAACGTAAAATGCG
7.8kb 3′UTR	GACTCGCTAGCAATGTTTTCAAGCCTATGGAGTG	GTAGTGCTAGCGGATCCTATTGTCATTAATATGATCTTTAATAAAC
BMPR2 full seq.		
Exon 1	GCCGGTCTACTTCCCATATT	CGAAGGGCAAGCACAGG
Exon 2	TTTGTCATTCCTTTATTTCCTTT	AACACAGTCATTTCAGGTAAGG
Exon 3	TCTTTATCATATTGTCTCCTTTTT	GGAAATACAAAGAAAAGTTGGTT
Exon 4	TGACATTTCAAAATTTGTTTTC	CGGAATTTAAAAGGAGCAAA
Exon 5	TTCTGCAGCTCTTCTTTTTAAG	TCACAGTAGAAACAACAGTCCAT
Exon 6.1	GAGAGCTGTAGCATTCTGTTT	TTGTCATGTTCCATCAAAGG
Exon 6.2	CCTTTGATGGAACATGACAA	AAGTGATCCACCTGCCTTAG
Exon 7	TGCAAATTCTTTATAAGGATGC	CCCACATGAGTGTCAATTTC
Exon 8	TTTCATGTTCAATAGTCCCTTTT	CATCAGTGTGATACCTTTTGT
Exon 9	TCAGAATATGCTACGTTCTCTCTC	TAACTGCTTCACTTCAAAAA
Exon 10	GAAATTTTATTCTGTCATTCTTTTC	TTCAGTCATAAGTCCTCTCTTT
Exon 11	TTTAAAGACACATGGTTTGACAT	ATAGATGCCACACCCCTTAG
Exon 12.1	TCATAAATGTACGTTCTCAATGTG	TTGTGCTTGCTGTCGTTC
Exon 12.2	GCACACCTTTGACTATAGGG	TGTAAGCAGACAGGGGTTG
Exon 12.3	ACCACAAATGTTGCACAGTC	CTGCTGTCCAGTTGCTTCTA
Exon 12.4	AGTTCTAGCTTGCTTTACCC	CAAGTTTGATTTGTGCTTGC
Exon 12.5	TAGTTTGCCTTTGAACACCA	TGGTTGTTTGGCCAGATAGT
Exon 12.6	ACAACCCAATATGCCAATG	TAGTTCGGCCACCTTCTAGT
Exon 12.7	GAAGGTGTTCTGGATCGTCT	CTGTATACTGCTGCCATCCA
Exon 12.8	ACAAGATGTTCTTGCACAGG	TTATTTAAATGGCCCCAAAA
Exon 13.1	CATCCCTTACCCGTTATTTC	GGAATGAACTGCCCTGTTAC
Exon 13.2	CTGGGTCATCTCCACTGAAT	GCATGTTTAAATGATGCAAAA