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

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CLINICAL–ALIMENTARY TRACT

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.**

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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

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