# Transforming Growth Factor β Signaling in Colorectal Cancer Cells With Microsatellite Instability Despite Biallelic Mutations in *TGFBR2*

Noel F. C. C. de Miranda,[1,§] Maarten van Dinther,[2,§] Brendy E. W. M. van den Akker,[1] Tom van Wezel,[1] Peter ten Dijke,[2,3,§] and Hans Morreau[1,§]

[1]Department of Pathology, [2]Department of Molecular Cell Biology, Cancer Genomics Centre Netherlands, Leiden University Medical Center, Leiden, The Netherlands; [3]Ludwig Institute for Cancer Research, Science for Life Laboratory, Uppsala University, Uppsala, Sweden

**BACKGROUND & AIMS:** Most colorectal cancer (CRC) cells with high levels of microsatellite instability (MSI-H) accumulate mutations at a microsatellite sequence in the gene encoding transforming growth factor β receptor II (*TGFBR2*). TGFβ signaling therefore is believed to be defective in these tumors, although CRC cells with *TGFBR2* mutations have been reported to remain sensitive to TGFβ. We investigated how TGFβ signaling might continue in MSI-H CRC cells. **METHODS:** We sequenced the 10-adenines microsatellite sequence in the *TGFBR2* gene of 32 MSI-H colon cancer tissues and 6 cell lines (HCT116, LS180, LS411N, RKO, SW48, and SW837). Activation of TGFβ signaling was detected by SMAD2 phosphorylation and through use of a TGFβ–responsive reporter construct in all CRC cell lines. Transcripts of *TGFBR2* were knocked-down in CRC cells using short hairpin RNA. Full-length and mutant forms of *TGFBR2* were expressed in LS411N cells, which do not respond to TGFβ, and their activities were measured. **RESULTS:** SMAD2 was phosphorylated in most MSI-H CRC tissues (strong detection in 44% and weak detection in 34% of MSI-H tumors). Phosphorylation of SMAD2 in MSI-H cells required *TGFBR2*—even the form encoding a frameshift mutation. Transcription and translation of *TGFBR2* with a 1-nucleotide deletion at its microsatellite sequence still produced a full-length TGFBR2 protein. However, protein expression required preservation of the *TGFBR2* microsatellite sequence; cells in which this sequence was replaced with a synonymous nonmicrosatellite sequence did not produce functional TGFBR2 protein. **CONCLUSION:** TGFβ signaling remains active in some MSI-H CRC cells despite the presence of frameshift mutations in the *TGFBR2* gene because the mutated gene still expresses a functional protein. Strategies to reactivate TGFβ signaling in colorectal tumors might not be warranted, and the functional effects of mutations at other regions of microsatellite instability should be evaluated.

*Keywords:* Colon Cancer; Signal Transduction; Tumor Suppressor; RNA Editing.

The transforming growth factor β (TGFβ) signaling pathway is an important mediator of tissue homeostasis.[1,2] It is believed that tumor cells target TGFβ signaling components to selectively escape its cytostatic effects.[3,4] Paradoxically, activation of TGFβ also has been associated with increased malignant behavior and metastatic capacity of cancer cells.[5,6]

TGFβ initiates its cellular responses by binding a TGFβ type 2 serine/threonine kinase transmembrane receptor, termed *TGFBR2*.[7] This interaction is facilitated by TGFβ type 3 receptor (TGFBR3), a co-receptor lacking enzymatic activity.[8] Upon ligand binding, TGFBR2 attracts and phosphorylates an additional serine/threonine kinase receptor, TGFBR1, which in turn phosphorylates the transcription factors SMAD2 and SMAD3.[9,10] When activated, SMAD2 and SMAD3 associate with the co-SMAD, SMAD4, and this complex accumulates in the cell nucleus where it induces or represses expression of selective genes.[11]

Approximately 15% of colorectal cancers (CRCs) present a microsatellite instability-high (MSI-H) phenotype, derived from a defective DNA mismatch-repair system.[12] MSI-H CRCs are characterized by the genome-wide accumulation of nucleotide insertions and deletions at microsatellite sequences. The third exon of the *TGFBR2* gene contains a 10-adenine microsatellite sequence (A10) that is mutated in the majority of MSI-H CRC tumors.[13,14] Nucleotide deletions and insertions at the 10A microsatellite produce frameshift mutations that lead to truncated nonfunctional TGFBR2 proteins.[15,16] Mutations also are common in the *ACVR2A* gene, which encodes an alternative type 2 TGFβ receptor and contains 2 microsatellite sequences at its 3rd and 10th exons.[17] Defects in other components of the TGFβ pathway include the following: loss of genetic material at the chromosome region 18q21.1, where *SMAD2* and *SMAD4* are located, and mutations in *SMAD4* in CRCs showing chromosomal instability[18,19]; mutations in *SMAD3* in approximately 4% of all CRCs[20]; and mutations in the 3'-untranslated region of the *BMPR2* gene in the majority of MSI-H CRCs.[21] Furthermore, pathogenic germline mutations in the *SMAD4* and *BMPR1A* genes are causative of juvenile polyposis.[19,22,23]

§Authors share co-senior authorship.

*Abbreviations used in this paper:* A10, 10 adenines microsatellite; cDNA, complementary DNA; CRC, colorectal cancer; MSI-H, microsatellite instability-high; p-SMAD2, phosphorylated SMAD2 protein; PCR, polymerase chain reaction; PEI, _____; qPCR, quantitative polymerase chain reaction; TGFβ, transforming growth factor β; TGFBR2, transforming growth factor β type 2 serine/threonine kinase transmembrane receptor.


0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2015.02.052

Because the availability of either TGFBR2 or ACVR2A is considered crucial for TGF$\beta$ signaling, the latter is believed to be defective in the majority of MSI-H CRC tumors. Nevertheless, few reports have indicated that cells carrying biallelic truncating mutations in *TGFBR2* and *ACVR2A* remain sensitive to TGF$\beta$ stimulation in vitro, but no mechanistic explanation for these observations has been provided.[24–27] In this article, we sought to explain the basis of retained TGF$\beta$ sensitivity in *TGFBR2*-mutated CRC cells.

## Materials and Methods

### Patient Material

A tissue microarray containing 76 formalin-fixed, paraffin-embedded, right-sided colon cancer tissues was used for immunohistochemical procedures. Thirty-two cases were classified as MSI-H according to the recommendations of the National Cancer Institute/ICG-HNPCC.[28] Formalin-fixed, paraffin-embedded tissues derived from all MSI-H tumors were processed further for flow sorting of tumor and stromal fractions. Fourteen nonmalignant colorectal tissues derived from CRC patients also were analyzed by immunohistochemistry. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (protocol P01-019).

### Cell Lines

Six CRC cell lines were obtained from the American Type Culture Collection: 5 MSI-H (HCT116, LS180, LS411N, RKO, and SW48) and 1 microsatellite-stable cell line (SW837). Cell identity was confirmed with the Cell ID System (Promega, Madison, WI). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and 100 U/mL of penicillin/streptomycin (Gibco, Life Technologies). The cells 293T and COS-1 were used for the production of lentiviral particles and/or for the transfection of *TGFBR2* constructs.

### Immunohistochemistry

A standard 2-step immunohistochemistry protocol was used for the detection of phosphorylated SMAD2 (p-SMAD2), SMAD4, and TMEPAI. Heat-mediated antigen retrieval was performed in 10 mmol/L citrate buffer solution (pH 6) for p-SMAD2 and TMEPAI or in 10 mmol/L Tris, 1 mmol/L EDTA solution (pH 9) for SMAD4 detection. Tissue sections were incubated overnight with the following primary antibodies: clone 138D4 (1:200; Cell Signaling Technology), which recognizes SMAD2 when phosphorylated at Ser465/467; clone B-8 (1:400; Santa Cruz Biotechnology) directed against SMAD4; and the ab128006 anti-TMEPAI rabbit polyclonal (1:800; Abcam). The following day, tissue sections were incubated for 30 minutes with Powervision Poly–horseradish peroxidase solution (Immunologic) and immunohistochemical detection was developed with the 3,3′-diaminobenzidine tetra hydrochloride-positive chromogen system (DAKO) for 5 minutes. Three scoring categories were used: negative, weak, and strongly positive, to which the numeric values of 0, 1, and 2 were assigned for statistical analyses. Validation of p-SMAD2 immunohistochemical detection was performed in SW837 cells (Supplementary Figure 1).

### Flow Sorting of Tumor and Normal Fractions

A flow-sorting procedure was used to genotype *TGFBR2* and *ACVR2A* mutations in separate fractions of tumor and stromal cells. Tissue preparation and flow cytometry analysis were performed as described previously.[29] Detailed procedures are included as Supplementary material.

### TGFBR2 and ACVR2A Fragment Analysis in Flow-Sorted Tumor Cells

To detect instability at the *TGFBR2* and *ACVR2A* microsatellite sequences, primers around *TGFBR2*'s 3rd exon, *ACVR2A*'s 3rd exon, and *ACVR2A*'s 10th exon were designed (primer sequences available upon request). Forward primers were 5'-end labeled with 6-HEX, and a "linker" sequence (5'-GTTTCTT) was added to the reverse primer. Polymerase chain reaction (PCR) amplifications were performed with 10 ng of DNA in iQ Supermix (Bio-Rad). PCR products were mixed in a formamide solution containing GeneScan 500 ROX Size Standard (Applied Biosystems). Thereafter, samples were loaded in a 4-capillary 3130 DNA Analyzer and results were interpreted with the GeneMapper 4.1 software (Applied Biosystems).

### TGFβ Pathway Reporter Transfection and Activity Measurement

CRC cell lines were seeded in 24-well plates and transiently transfected with 700 ng of a $(CAGA)_{12}$-luciferase reporter construct,[30,31] and 300 ng of a cytomegalovirus-LacZ expression plasmid (Clontech). For *TGFBR2* overexpression in LS411N cells, 300 ng of the $(CAGA)_{12}$-luciferase reporter construct, 200 ng of the cytomegalovirus-LacZ construct, and 10 ng of a pcDNA3-Myc-TGFBR2 plasmid were used. Cells were transfected using Lipofectamine 2000 (Life Technologies), according to the manufacturer's protocol. After 2 days, cells were serum-starved for 8 hours and stimulated for 16 hours with TGF$\beta$3 (kindly provided by Professor Andrew P. Hinck, University of Wisconsin, WI). Lysis and measurement of luciferase activity were performed with the Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions using $\beta$-galactosidase activity as an internal control.

### TGFβ Stimulation and p-SMAD2 Detection in CRC Cells With TGFBR2 Stable Knockdowns

Detailed procedures for the generation of *TGFBR2* stable knockdowns are included as Supplementary material. To detect SMAD2 phosphorylation in control and *TGFBR2* knockdown cells, cells were cultured in 6-well plates, serum-starved overnight, and incubated with various concentrations of TGF$\beta$3 for 1 hour before lysis. Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and, after blotting, proteins were detected with anti–p-SMAD2 (rabbit polyclonal #3101; Cell Signaling), anti-SMAD2 (clone 18/Smad2/3; BD Biosciences), and anti–glyceraldehyde-3-phosphate dehydrogenase (clone 6C5; Chemicon) antibodies.

### Expression of TGFβ Target Genes

Real-time quantitative PCR (qPCR) was used to measure the expression of 18 TGF$\beta$ target genes in CRC cell lines, namely: *PAI-1*, *TMEPAI*, *SMAD7*, *PDGFA*, *TLGLN2*, *CTGF*, *MYC*,