Loss of Heterozygosity and Mutational Analyses of the *ACTRII* Gene Locus in Human Colorectal Tumors

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SUMMARY: The *activin type II receptor* gene (*ACTRII*) is mutated in 58.1% of microsatellite-unstable (MSI-H) colorectal cancers and is a close relative of the TGFβ-1 type II receptor, which is known to be involved in both MSI-H and non–MSI-H colorectal carcinogenesis. We therefore sought to determine whether *ACTRII* was involved in non–MSI-H colorectal cancers. We evaluated *ACTRII* inactivation by allelic deletion, loss of mRNA expression, or somatic mutation in 51 non–MSI-H colon cancers. Loss of heterozygosity (LOH) at the *ACTRII* locus (2q23.1) was found in 9 (17.6%) of 51 primary tumors. Loss of *ACTRII* mRNA expression was seen in one (14.3%) of the seven LOH-positive primary tumors from which total RNA was available. We also performed DNA sequencing analysis of tumors showing LOH. One LOH-positive primary tumor exhibited a novel germline missense sequence alteration (amino acid substitution, 117 Ile to Phe) that was not found in 23 additional normal individuals, implying that this alteration is not a frequent polymorphism. We conclude that *ACTRII* is probably involved in both non–MSI-H and MSI-H colorectal carcinogenesis, but more frequently in the latter subgroup. (*Lab Invest 2003, 83:1867–1871*).

*C*olorectal cancer is the second most common cause of cancer-related deaths in the United States and the third most common cancer in both genders, with an estimated 135,400 new cases and 56,700 deaths occurring in the year 2001 (American Cancer Society, 2001). Therefore, it is crucial to discover and better understand molecular pathways underlying this type of cancer, including the identification of new tumor suppressor genes as well as mechanisms involved in their inactivation.

The *activin type II receptor* gene (*ACTRII*) was identified by genome-wide frameshift mutational screening as a novel candidate tumor suppressor gene mutated very frequently (in 58.1% of MSI-H colorectal cancers, ie, tumors with high microsatellite instability) at an $(A)_8$ mononucleotide repeat within its coding region (Mori et al, 2001). ACTRII is a member of the transforming growth factor β (TGF-β) receptor family and is associated with the activin-SMAD signaling pathway, which is in turn involved in the induction of differentiation, growth suppression, and apoptosis in a variety of cell types, including those arising from the intestinal epithelium (Attisano et al, 1996;

Sonoyama et al, 2000). Interference with the *ACTRII* signaling pathway in vitro inhibits cell differentiation (Li et al, 1998; Liu et al, 2000) and apoptosis (Chen et al, 2000; Choi et al, 2001). The TGF-β–SMAD signaling pathway, which shares the downstream effectors SMAD2 and SMAD4 with the activin-SMAD pathway, is reported to be responsible for human tumor suppression (Attisano et al, 1996; Liu et al, 2000; Sonoyama et al, 2000; Su et al, 2001). ACTRII forms an active heterodimeric receptor complex with ACTRI (Attisano et al, 1993). Of interest, somatic mutation of *ACTRI* was recently described in pancreatic carcinoma (Su et al, 2001).

To investigate whether *ACTRII* was involved in all colorectal tumors, rather than just those with frequent microsatellite instability, we studied inactivation of *ACTRII* in non–MSI-H tumors. We performed detailed loss of heterozygosity (LOH) studies of 2q22.1-q23.3, the region containing the *ACTRII* gene, in 51 non–MSI-H colorectal tumors. LOH-positive tumors were further tested for somatic point mutations within *ACTRII*.

Results

Allelotyping revealed that 17 (33%) of 51 colorectal tumors showed LOH at one or more of four loci on chromosome 2q23.1. These results are summarized in Table 1. Locus D2S141 showed the highest rate of LOH (12 of 27 informative cases or 44.4%). Nine (17.6%) of the 51 tumors manifested LOH flanking the *ACTRII* locus: these comprised 7 adenocarcinomas

Table 1. LOH Mapping Results at *ACTRII* Locus

Position	2q22.1	2q23.1	*ACTR2*	2q23.3	2q24.1
Primer	D2S127	D2S151		D2S2299	D2S141
987	LOH	N/A		LOH	LOH
1008	—	LOH		N/A	LOH
13687	LOH	N/A		—	N/A
13799	LOH	—		N/A	LOH
14127	N/A	—		LOH	N/A
14062	LOH	N/A		—	—
14280	—	N/A		N/A	LOH
14401	LOH	—		LOH	N/A
12385	LOH	LOH			LOH
LOH/informative	8/36	2/37		3/38	12/27
% LOH	22.22%	5.40%		7.89%	44.44%

2q allelotyping results for the nine tumors showing LOH at the *ACTRII* locus (2q22.1-q23.3). LOH, loss of heterozygosity; (−), LOH negative; N/A, noninformative. Summarized results for all 52 tumors are shown in the bottom rows. The four microsatellite markers are ordered according to their position on the chromosome arm 2q (ie, left=centromeric side, right=telomeric side). The position of the *ACTRII* gene is as indicated. LOH was determined by comparing electrophoretic profiles of PCR products from tumor and corresponding normal genomic DNAs on an automated DNA sequencing apparatus (MegaBace 1000; Molecular Dynamics). Genomic DNA was extracted from frozen tissues by standard protocols (Attisano et al, 1993). PCR conditions were as described previously (Mori et al, 2001).

and 2 adenomas, one of which contained focal high-grade dysplasia.

Quantitative RT-PCR revealed one tumor totally lacking *ACTRII* mRNA expression (Fig. 1). This finding was confirmed with multiple repeat assays. DNA sequencing of the remaining six tumors from which RNA was available revealed one containing a single-nucleotide change (TTT to ATT, amino acid substitution Ile to Phe) at codon 117 in the extracellular domain of ACTRII. This sequence alteration also appeared in the corresponding normal sample (Fig. 2). In addition, a silent polymorphism at codon 118 (CCG to CCA) was discovered in 3 (50%) of the 6 primary tumor samples; this polymorphism was then found to have been previously described (D'Abronzo et al, 1999). This polymorphism was also observed in five (62.5%) of the eight colorectal cancer cell lines we studied (D'Abronzo et al, 1999). Finally, among these eight cell lines, we detected a one-base deletion within
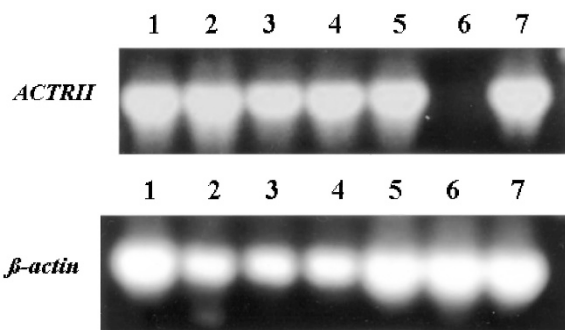

Figure 1.
ACTRII gene expression in tumors with LOH. RT-PCR analysis of the loss of heterozygosity (LOH)+ samples. This figure shows the gel picture of the RT-PCR product for seven LOH-positive tumors. The results for *β-actin* RT-PCR are presented as an internal control. The RT-PCR was performed according to the Invitrogen/Life Technologies protocol. PCR conditions were described previously (Mori et al, 2001). *Top*, The 1100-bp fragment of *ACTRII* gene; *bottom*, the 436-bp RT-PCR product of the *β-actin* gene. *Lane 6* presents total lack of mRNA expression of the *ACTRII* gene.

an (A)$_8$ repeat at codon 437 in SW48 cells, which are known to be MSI-H (Branch et al, 1995).

Discussion

ACTRII is frequently mutated in MSI-H colorectal cancers (Mori et al, 2001). Moreover, activin is known to play a role in intestinal cell growth and differentiation (Attisano et al, 1996; Sonoyama et al, 2000). Finally, there are strong structural and functional similarities between the activin and TGF-$β$ receptors (Attisano et al, 1993), the latter of which is known to be involved in *both* MSI-H and non–MSI-H colorectal tumorigenesis (Calin et al, 2000; Grady et al, 1998, 1999; Orimo et al, 1998). For these reasons, we studied somatic alterations of the *ACTRII* gene in non–MSI-H colorectal cancers. The occurrence of allelic deletion at 2q23.1 in two adenomas (one with focal-grade dysplasia), as well as in seven adenocarcinomas, suggests that LOH at this locus may occur early in colorectal neoplasia.

To evaluate whether deletion of one allele of *ACTRII* was associated with inactivation of the remaining allele, we performed DNA sequencing of the entire coding region of *ACTRII* in seven primary LOH-positive tumors from which RNA was available, as well as in eight colorectal cancer cell lines. This mutational analysis revealed, in addition to a silent polymorphism at codon 118 in five of eight cell lines and three of six tumors showing ACTRII expression, a single patient with a novel alteration at codon 117 in both tumor and corresponding normal samples.

Notably, the codon 117 alteration (I117F) found in one primary tumor has not been previously described. To determine whether I117F represented a germline mutation or a polymorphism, we sequenced normal genomic DNA from 23 additional individuals, focusing on the second exon of the *ACTRII* gene (containing codon 117). None of these individuals possessed this alteration, suggesting that I117F is not a frequent polymorphism in the general population.
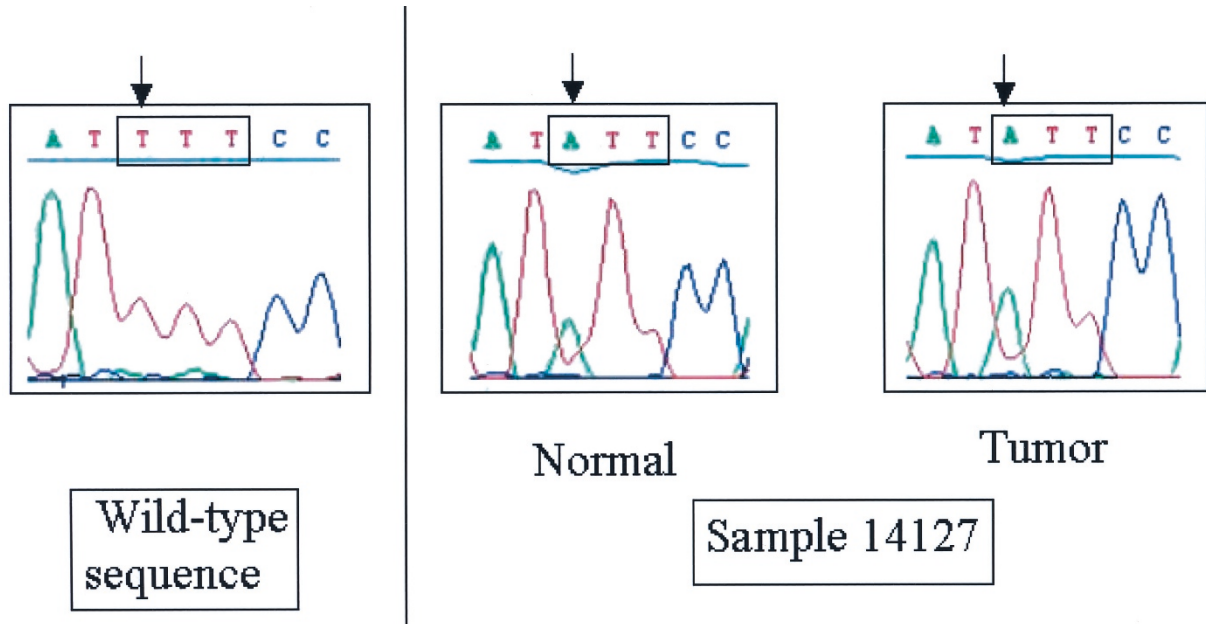
Figure 2.
Germline sequence alteration in *ACTRII*. Nucleotide change at codon 117. Nucleotide change from normal T (*left*) to A (*right*) at codon 117, resulting in amino acid change from Ile to Phe in the extracellular domain of the receptor. The figures show the sense sequence, and the codon 117 is marked in both panels. An *arrow* indicates the mutated nucleotide.

To explore the involvement of ACTRII codon 117 in human tumorigenesis in general, we performed a literature search. Three amino acids within the ACTRII extracellular domain, 42F, 60W, and 83F, are known to be involved in the binding of activin and inhibin to ACTRII: mutations of these residues in the full-length receptor cause disruption of activin and inhibin binding (Gray et al, 2000).

Complete lack of *ACTRII* mRNA expression was discovered in one of seven LOH-positive primary non–MSI-H tumors but in none of eight colorectal cancer cell lines. It is possible that promoter hypermethylation caused this lack of expression; however, a promoter region for ACTRII could not be identified in public DNA sequence databases, nor were upstream CpG islands found at the ACTRII genomic locus. Finally, a mutation at codon 437, resulting in a predicted truncated protein, was observed in SW48, the only MSI-H colorectal cancer cell line studied.

These data imply that genetic alterations of the *ACTRII* gene (ie, missense mutation, total loss of mRNA expression, and LOH) occur in non–MSI-H colorectal cancers, but at a lower frequency than in MSI-H cancers. Thus, events predicted to result in *ACTRII* inactivation occur in both MSI-H and non–MSI-H colorectal tumors, further supporting the candidacy of *ACTRII* as a colorectal tumor suppressor gene.

Materials and Methods

Tissue Preparation, DNA, and RNA Extraction

Fifty-one non–MSI-H colorectal cancers showing MSI at no more than one of five consensus loci (BAT 25, BAT 26, D2S123, D5S346, and D17S250) were obtained with matching normal control tissues. All specimens were harvested at the time of surgical procedures or endoscopies and immediately frozen in liquid nitrogen. DNA and RNA were extracted from frozen tissue samples using a DNeasy Tissue Kit and RNeasy Mini Kit from Qiagen (Valencia, California), following the manufacturer's protocol.

Alellotyping Analysis

For the LOH study, four microsatellite markers localizing within a 17-Mb region at 2q22.1-q23.3 including the *ACTRII* gene (2q23.1) were used: D2S127, D2S151, D2S2299, and D2S141 (Table 2). The *ACTRII* gene is located between D2S151 and D2S2299. PCR reactions were performed in a total volume of 10 μl containing 20 ng of genomic DNA, 0.5 μM each primer, 1× *Taq* polymerase buffer (Life Technologies, Gaithersburg, Maryland), 0.1 mM each dNTP, 1.5 mM MgCl, and 0.1 IU of *Taq*DNA polymerase (Life Technologies). PCR conditions were described previously (Mori et al, 2001). Ten microliters of each PCR product were analyzed on an automated DNA sequencer (MegaBace 1000; Amersham LifeSciences/Molecular Dynamics, Sunnyvale, California) using the software program Genetic Profiler, Version 1.0 (Amersham Life Sciences/Molecular Dynamics). We classified a tumor-specific alteration as LOH when it resulted in a change of more than 50% in tumor allele peak height relative to the corresponding normal.

Mutational Analyses

RT-PCR for Expression Levels and cDNA Sequencing. RNA was available from seven of the LOH-positive

Table 2. Details of the Four Microsatellite Markers Used in LOH Mapping at 2q22.1-q23.3

Locus	Position	Size range	Sequence
D2S127	2q21-q33	282–303	5′-GTT GCT GCA AAT GGC AGG ATT-3′ (sense)
			5′-CCA CCC AAA CCT AAC AGA TA-3′ (antisense)
D2S151	2q21-q33	211–229	5′-GAA GCC CTG ACT TAT GAA CTA-3′ (sense)
			5′-CCA ATT CAG TGG CAT CTA TG-3′ (antisense)
D2S2299	2qter-pter	272–294	5′-GTT GCT GCA AAT GGC AGG ATT-3′ (sense)
			5′-ATG TGG AAC TGT GAG TCA ATT A-3′ (antisense)
D2S141	2q21-q33	152–178	5′-CCA CAC TCC CCA GCC AC-3′ (sense)
			5′-CCA AAC ACA TAC AGT GAA CTT C-3′ (antisense)

Four microsatellite markers (D2S127, D2S151, D2S2299 and D2S141) localized within a 17-Mb region on chromosome arm 2q22.1-q23.3 were used in this study. The *ACTRII* gene is located on 2q23.1, between D2S151 and D2S2299, which are located within 6 Mb distance from each other.

tumors, as well as from eight colorectal cancer cell lines (SW480, SW48, Colo320HSR, WiDr, SW620, SW948, Colo201, and Colo32DM). Reverse transcription used 10 μg of total mRNA in a total reaction volume of 20 μl, containing 0.5 μg of Oligo dT (Invitrogen, Carlsbad, California), 0.1 mM dNTP mix (Invitrogen), 4 μl of 5× First-Strand Buffer (ibid), 2 μl 0.1 M dithiothreitol (Invitrogen), and 1 μl of RNase OUT Recombinant Ribonuclease Inhibitor 40 U/μl (Invitrogen). PCR protocol was performed in a total volume of 20 μl, containing 1.75 μl of primer (Invitrogen), 0.1 mM dA/T/C/GTP, 0.1 μl of *Taq*DNA polymerase (Qiagen), and 10× *Taq* polymerase buffer (Qiagen) under the same conditions as described in allelotyping analysis. Internal control RT-PCR for the β-actin gene consisted of the same protocol, with the primers 5'-CCA GAG CAA GAG AGG TAT CC-3' and 5'-CTG TGG TGG TGA AGC TGT AG-3'. PCR product electrophoresis was performed on a 1.5% agarose gel. Sequencing analysis was performed on both sense and antisense strands of cDNA obtained by RT-PCR.

cDNA Sequence Analysis. Sequencing reactions were performed using the DYEnamic ET terminator kit (Amersham Pharmacia Biotech) following the manufacturer's protocol. Electrophoresis was performed on a MegaBace 1000 (Molecular Dynamics) and analyzed using Sequence Analyzer software, Version 2.0 (Amersham Pharmacia Biotech). DNA sequences from tumors or cell lines were compared with the wild-type *ACTRII* sequence retrieved from the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). Genomic DNA from 24 colorectal cancers was sequenced at the second exon of the *ACTRII* gene in accordance with the protocol described above, using primers 5'-TTC TCT GCT TAT TTA TAG GAC TG-3'and 5'-GTT TCC AAT CTA CAG TTG AGC-3'.

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