SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway

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Loss of chromosome 18q21 is well documented in colorectal cancer, and it has been suggested that this loss targets the *DCC*, *DPC4*/*SMAD4*, and *SMAD2* genes. Recently, the importance of *SMAD4*, a downstream regulator in the TGF-β signaling pathway, in colorectal cancer has been highlighted, although the frequency of *SMAD4* mutations appears much lower than that of 18q21 loss. We set out to investigate allele loss, mutations, protein expression, and cytogenetics of chromosome 18 copy number in a collection of 44 colorectal cancer cell lines of known status with respect to microsatellite instability (MSI). Fourteen of thirty-two MSI$^-$ lines showed loss of SMAD4 protein expression; usually, one allele was lost and the other was mutated in one of a number of ways, including deletions of various sizes, splice site changes, and missense and nonsense point mutations (although no frameshifts). Of the 18 MSI$^-$ cancers with retained SMAD4 expression, four harbored missense mutations in the 3′ part of the gene and showed allele loss. The remaining 14 MSI$^-$ lines had no detectable *SMAD4* mutation, but all showed allele loss at *SMAD4* and/or *DCC*. *SMAD4* mutations can therefore account for about 50–60% of the 18q21 allele loss in colorectal cancer. No MSI$^+$ cancer showed loss of SMAD4 protein or *SMAD4* mutation, and very few had allelic loss at *SMAD4* or *DCC*, although many of these MSI$^+$ lines did carry *TGFBIIR* changes. Although *SMAD4* mutations have been associated with late-stage or metastatic disease, our combined molecular and cytogenetic data best fit a model in which *SMAD4* mutations occur before colorectal cancers become aneuploid/polyploid, but after the MSI$^+$ and MSI$^-$ pathways diverge. Thus, MSI$^+$ cancers may diverge first, followed by CIN$^+$ (chromosomal instability) cancers, leaving other cancers to follow a CIN$^-$MSI$^-$ pathway.

colorectal cancer cell lines | 18q

Allele loss at 18q21.1 has been demonstrated in up to 60% of colorectal cancers (CRCs; ref. 1). Mapping to this chromosomal band are *DCC* (*D*eleted in *C*olon *C*arcinoma), *DPC4*/*SMAD4* (*D*eleted in *P*ancreatic *C*ancer *4*) and *SMAD2*. For many years the allele loss observed around 18q21.1 in colorectal cancer was believed to be targeting *DCC* (2, 3). It has more recently been shown, however, that mutations of *DCC* and *SMAD2* are very rare in colorectal cancer (4). By contrast, changes in *SMAD4* are much more common in the pathogenesis and evolution of colorectal cancer (5, 6), as has also been shown in pancreatic cancer (7). In particular, inactivation of *SMAD4* has been associated with late stage or metastatic colorectal cancer (6, 8, 9). Additionally, it has recently been shown that constitutional mutations in *SMAD4* can cause Juvenile Polyposis Syndrome (JPS; refs. 10 and 11), a disorder characterized by the presence of hamartomatous polyps in the gastrointestinal tract and a markedly increased risk of developing a gastrointestinal malignancy.

The *SMAD4* gene codes for a protein involved as a downstream regulator in the transforming growth factor β (TGF-β) signal transduction pathway. The SMAD4 protein acts as a trimer and forms complexes with the receptor-phosphorylated SMAD2 and SMAD3; these heteromeric complexes then translocate from the cytoplasm to the nucleus where association with DNA binding factors facilitates the transcription of target genes. These target genes include cyclin-dependent kinase inhibitors such as p15(ink4B) (12) and the inhibitory SMAD7 (13, 14). Abrogation of SMAD4 function may cause a breakdown in this signaling pathway and loss of transcription of genes critical to cell-cycle control (15). Cells may therefore become TGF-β resistant and escape from TGF-β-mediated growth control and apoptosis (16).

Loss of 18q21 is seen in 80% of pancreatic cancers, with inactivation of *SMAD4* shown to result from homozygous deletions in 30% of cases and point mutations/small alterations in 20% of cases (7). Of the 60% of colorectal cancers showing 18q loss, mutations of *SMAD4* have been demonstrated in about one third, with the loss thought to be targeting *DCC* or another nearby gene in the remainder of cases (1).

We have determined how many of 44 colorectal cancer cell lines have loss of the SMAD4 protein and investigated the cause of this loss by mutation analysis and assessment of allele loss. In a subset of lines, we have also determined the copy number of *SMAD4*, using molecular cytogenetic techniques, and related this to the karyotype of the cell line. The results have implications not just for *SMAD4* inactivation in bowel tumors, but also more generally for genetic pathways of colorectal tumorigenesis.

Materials and Methods

DNA was extracted from 44 established CRC cell lines (C10, C32, C70, C80, C84, C99, C106, C125, CAC02, COLO201, COLO205, COLO320, COLO678, COLO741, CX1/HT29, GP2D, HCA46, HCA7, HCT8, DLD1/HCT15, HCT116, HRA19, HT55, LIM1863, LOVO, LS174T, LS180, LS411, LS1034, PC/JW, SKC01, SW48, SW403, SW480, SW620, SW837, SW948, SW1116, SW1222, SW1417, VACO4A, VACO4S, VACO5, and VACO10), using standard methods. The *APC* mutation status, β-catenin mutation status, and microsatellite instability (MSI) status have been reported (summarized in ref. 17).

For F-SSCP (fluorescent-single stranded conformational polymorphism analysis), exon-by-exon amplification of the 11 exons of *SMAD4* (covering all coding sequence and intron/exon boundaries) was performed in each CRC line by using previously reported primers (11) with added fluorescent 5′ and 3′ labels (FAM, HEX,

Abbreviations: MSI, microsatellite instability; CRC, colorectal cancer; CIN, chromosomal instability.

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or TET). PCRs were then diluted 1:50 with distilled water and combined with an internal size standard (Tamra 500, Perkin–Elmer Applied Biosystems) and formamide. F-SSCP analysis at 20°C was performed by using an ABI310 sequencer (Perkin–Elmer Applied Biosystems). Fragments showing both aberrant and normal migration were reamplified by using non-fluorescently labeled primers, purified by using Qiaquick columns (Qiagen, Hilden, Germany) and then sequenced in both forward and reverse orientations by using the ABI Big Dye Terminator kit (Perkin–Elmer Applied Biosystems). Repeated failure to amplify any segment of the *SMAD4* gene in the PCR, despite successful amplification in control PCRs (17), was taken to denote homozygous deletion of that segment.

For Western blotting, pellets from all 44 CRC cell lines were lysed in 0.1 M DTT/bromophenol blue and separated on a 15% resolving gel. After transfer to the poly(vinylidene difluoride) (PVDF) membrane (Millipore) and blocking in 5% Marvel (Premier Brands, Stafford, United Kingdom), the primary antibodies were exposed to the membrane for 2 hours at room temperature. The anti-SMAD4 mouse monoclonal antibody B8 (Santa Cruz Biotechnology) recognizes an epitope in exon 5 (codons 68–108; M. Howell and C. Hill, personal communication); this antibody was diluted 1/100, and the control anti-β-actin mouse monoclonal antibody (Sigma) was diluted 1/1,000, and exposed to the membrane both simultaneously and separately. After detection of the proteins with enhanced chemiluminescence reagents (ECL, Amersham Pharmacia) according to the manufacturer's instructions, the membrane was exposed to film for 1 and 5 min. After scanning the films by using a Bio-Rad GS-700 densitometer, band intensity ratios were compared for SMAD4 and β-actin. SMAD4 protein was classed simply as "present" or "absent," based on immunohistochemical data showing absent protein expression in many pancreatic cancers (18), colorectal cancers, and JPS polyps (K.L.W.-R. and I.P.M.T., unpublished data).

For the protein truncation test (PTT), RNA was extracted from a subset of CRC cell lines (SW1222, HRA19, SKC01, SW948, JW, HCT8, COLO205, LS174T, SW48, LOVO, SW620, COLO320, GP2D, HT29, CACO2, and HCA46) by using the Fast-track RNA extraction kit (Invitrogen). cDNA was synthesized by using the First Strand Synthesis kit (Promega). Primer pairs were designed by using Primer 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi) to cover the entire *SMAD4* coding sequence (GenBank accession no. U44378). PCRs were performed by using standard conditions with either primer pair A (forward 5′-atggacaatatgtctattacga-3′, reverse 5′-ggaatgcaagctcattgtga-3′) covering codons 1–311 or primer pair B (forward 5′-cagcatccaccaagtaatcg-3′, reverse 5′-aaggttgtgggtctgcaatc-3′) covering codons 182 to 553, with the forward primer tagged with a T7 RNA-polymerase binding site, a ribosome binding site, and an in-frame start codon. *In vitro* coupled transcription–translation was performed on the tagged PCR products by using the T^{N}T Rabbit Reticulocyte Lysate Kit (Promega), incorporating [^{35}S]methionine, and the resulting "proteins" were separated according to size on a 12.5% polyacrylamide resolving gel. Once fixed and dried, gels were exposed to film overnight and developed.

For the assessment of 18q allelic loss, seven microsatellite markers were selected from the Location Database (http://cedar.genetics.soton.ac.uk/pub/chrom18/map.html): for SMAD4 the markers were in the order, D18S479–1.45 Mb–D18S474–0.01 Mb–D18S46–0.35 Mb–(*SMAD4*); and for *DCC* (about 1.5 Mb telomeric of *SMAD4*) the order was D18S484–0.16 Mb–D18S487–0.44 Mb–DCC–0.19 Mb–D18S35. The heterozygosity for the seven markers was reported to be 60%, 82%, 80%, 72%, 81%, 87%, and 70%, respectively. In addition, markers mapping to 18p (D18S481) and the telomeric region of 18q (D18S878) were included. The forward primer was fluorescently labeled with HEX, FAM, or TET. Standard PCR conditions were used, and then the PCR products were combined with the internal size standard Tamra350 (Perkin–Elmer Applied Biosystems) and run on an ABI377 semiautomated sequencer. Results were analyzed by using GENOTYPER software to assign peak sizes. Because no normal tissue was available, allelic loss was assumed to have occurred at *DCC* or *SMAD4* if all microsatellite markers close to that gene were hemizygous/homozygous (corresponding to $P < 0.01$).

CRC cell line karyotypes were determined by using standard methods. Copy number of 18q21 was assessed by a combination of comparative genomic hybridization (CGH; refs. 19 and 20), spectral karyotyping (SKY; ref. 21), and locus-specific fluorescence *in situ* hybridization (FISH), using PAC 224 j 22, to which *SMAD4* is known to map, as described (22). The samples analyzed by using cytogenetic techniques are shown in Table 1.

Results

Overall, 32% of the CRC cell lines showed loss of SMAD4 expression (Table 1, Fig. 1), comprising 14 of 32 (44%) MSI$^-$ lines and 0 of 14 MSI$^+$ lines ($P < 0.004$, Fisher's exact test). In no case was a truncated protein band observed (Table 1). Several different types of mutation were found to account for the loss of expression (Table 1). Importantly, sequencing showed that all *SMAD4* mutations were present in the homozygous or hemizygous state (Fig. 2), with no underlying wild-type sequence.

For one cell line (COLO678), the whole gene was homozygously deleted, whereas other cell lines showed partial homozygous deletions (exons 1–4 in COLO201/COLO205, and exons 10–11 in SW403). All these mutations were accompanied by allelic loss at *SMAD4* (Table 1).

Putative splicing mutations were detected in three cell lines, SW480 and SW620 (derived from a primary tumor and metastasis) and C10. All these lines showed absent SMAD4 protein and allelic loss at *SMAD4*. Of these three lines, SW620 was tested by using the protein truncation test (PTT) and only normal-length mRNA was detected. Thus, although the mutation in SW620 does not lead to detectable abnormal mRNA splicing, we cannot exclude the possibility that it has pathogenic effects through reduction of normal mRNA levels, which could be assessed by using RNase protection assay.

Nonsense *SMAD4* mutations, accompanied by allele loss and absent protein, were detected in two cell lines: HT29/CX1 (Q311X, exon 7) and VACO10MS (Q239X, exon 5). Missense mutations, plus allelic loss, were found in four lines: CACO2 (D351H), C80 (D351H), SW948 (D537Y), and SW1222 (L540R). SMAD4 protein was present in all of these four lines.

Four MSI$^-$ cell lines had absent SMAD4 protein and allelic loss, but no detectable *SMAD4* mutation (Table 1). None of the 14 MSI$^+$ cell lines possessed a pathogenic *SMAD4* mutation, a highly significant difference from the MSI$^-$ lines ($P < 0.005$, Fisher's exact test). Known *SMAD4* intronic polymorphisms (23) were also detected (data not shown) in MSI$^-$ and MSI$^+$ lines.

Overall, there was a striking level of allele loss at *SMAD4* and *DCC* in all of the MSI$^-$ lines, with 30/32 (94%) showing loss at *SMAD4* and 30/32 (94%) with loss at *DCC* (Table 1). In accordance with the sequencing data, all lines with allelic loss showed complete absence of one microsatellite allele; thus, all copies of 18q21 in each cell were derived from the same chromosome-18 homologue, even where the cancer was polyploid and had more than one copy of 18q. In contrast, 11/12 (92%) MSI$^+$ lines showed heterozygosity for at least one marker each at *SMAD4* and *DCC*, indicating that loss of 18q material is not critical for the development and progression of these tumors. The different levels of 18q21 loss in MSI$^-$ and MSI$^+$ tumors were confirmed in a set of 61 sporadic colorectal tumors, in which mean loss at four markers close to *SMAD4* and *DCC* was 4% in 14 MSI$^+$ cancers and 53% in 47 MSI$^-$ cancers ($P < 0.003$, Fisher's exact test; data not shown).

Table 1. Summary of molecular and cytogenetic data for 18q21.1 status in colorectal cancer cell lines

Cell line	MSI*	B8 Western†	SMAD4 mutation‡	Predicted effect of mutation§	Loss at SMAD4¶	Loss at DCC‖	Karyotype**	18q21 status††	TGFBIIR‡‡
COLO678	MSI−	Absent	EX1 − 11del	No protein	Yes	Yes	?		—
COLO201	MSI−	Absent	EX1 − 4del	No protein	Yes	Yes	78	2 copies	None
COLO205	MSI−	Absent	EX1 − 4del	No protein	Yes	Yes	68	2 copies	None
VACO10	MSI−	Absent	c.715C>T (Q239X), EX5	Nonsense	Yes	Yes	115	—	None
C10	MSI−	Absent	IVS6 − 1G>T	Splice disruption	Yes	Yes	49	—	—
HT29/CX1	MSI−	Absent	c.931C>T (Q311X), EX7	Nonsense	2/3 markers	Yes	71	2 copies	None
SW480	MSI−	Absent	IVS7 + 5G>C	Splice disruption	Yes	Yes	57	1 copy	None
SW620	MSI−	Absent	IVS7 + 5G>C	Splice disruption	Yes	Yes	50	1 copy	None
CACO2	MSI−	Present	c.1051G>C (D351H), EX8	Missense	Yes	Yes	96	—	None
C80	MSI−	Present	c.1051G>C (D351H), EX8	Missense	Yes	Yes	69	2 copies	—
SW403	MSI−	Absent	EX10 − 11del	Truncated protein	Yes	Yes	68	2 copies	—
SW948	MSI−	Present	c.1609G>T (D537Y), EX11	Missense	Yes	No	67	—	—
SW1222	MSI−	Present	c.1619T>G (L540R), EX11	Missense	Yes	Yes	?	18q loss	—
SW1116	MSI−	Absent	None found		Yes	Yes	63	—	—
HT55	MSI−	Absent	None found		Yes	Yes	80	2 copies	—
C106	MSI−	Absent	None found		Yes	Yes	79	2 copies	—
PC/JW	MSI−	Absent	None found		Yes	Yes	70	2 copies	—
SW1417	MSI−	Absent	None found		Yes	No	70	2 copies	—
SW837	MSI−	Present	None found		Yes	Yes	40	1 copy	None
C99	MSI−	Present	None found		Yes	Yes	52	1 copy	—
C84	MSI−	Present	None found		No	Yes	56	1 copy	—
C125	MSI−	Present	None found		Yes	Yes	60	1 copy	—
VACO4A	MSI−	Present	None found		Yes	Yes	60	2 copies	—
HCA46	MSI−	Present	None found		Yes	Yes	71	2 copies	None
C32	MSI−	Present	None found		Yes	Yes	74	2 copies	—
LIM21-1863	MSI−	Present	None found		Yes	Yes	80	3 copies	—
C70	MSI−	Present	None found		Yes	Yes	127	4 copies	—
COLO320	MSI−	Present	None found		Yes	Yes	53	—	None
VACO4S	MSI−	Present	None found		Yes	Yes	64	—	—
LS1034	MSI−	Present	None found		Yes	Yes	77	—	—
HRA19	MSI−	Present	None found		Yes	Yes	?	—	—
SKC01	MSI−	Present	None found		No	Yes	hypertriploid	—	—
HCT8	MSI+	Present	None found		No	No	?	—	—
LS180	MSI+	Present	None found		No	No	45	—	—
SW48	MSI+	Present	None found		No	No	47	—	Mutant × 2
LS174T	MSI+	Present	None found		No	No	45	2 copies	Mutant × 2
DLD1/HCT15	MSI+	Present	None found		No	No	46	2 copies	Mutant × 2
LOVO	MSI+	Present	None found		No	No	49	2 copies	Mutant × 2
VACO5	MSI+	Present	None found		No	No	47	2 copies	Mutant × 2
HCA7	MSI+	Present	None found		No	No	43	2 copies	Mutant × 2
HCT116	MSI+	Present	None found		No	No	45	2 copies	Mutant × 2
LS411	MSI+	Present	None found		No	No	75	2 copies	Mutant × ?
GP2D	MSI+	Present	None found		No	No	46	2 copies	—
COLO741	MSI+	Present	None found		Yes	Yes	?	2 copies	—

COLO201/COLO205, HT29/CX1, and DLD1/HCT8/HCT15 are essentially identical cell lines.

*MSI status.

†SMAD4 protein expression as assessed by using B8 and western blotting.

‡Identified *SMAD4* mutations.

§Predicted effect of *SMAD4* mutations.

¶‖Allelic loss as inferred from homozygosity at microsatellite markers near *SMAD4* and at *DCC*, respectively.

**Modal chromosome number of cell line (?, not known).

††18q21.1 status as determined by CGH, SKY, and FISH (—, not done).

‡‡*TGFBIIR* mutation status (—, not done).

The molecular cytogenetic and karyotype data are summarized in Table 1. In the lines with 18q21 loss, the amount of chromosome 18q21 material was always less than the overall ploidy. For example MSI− cell lines with a modal chromosome number of less than 60 (C84, SW837, C99, C125, SW480, and SW620) had only one copy of chromosome 18q21, whereas cell lines with near-triploid karyotypes (SW403, HT29/CX1, HT55, COLO201, COLO205, C32, C106, C80, PC/JW, SW1417, VACO4A, and HCA46) had two copies of chromosome 18q21. Two lines with hypotetraploid (LIM1863) and hypohexaploid (C70) karyotypes had three and four copies of chromosome 18q21, respectively. Typing of extra markers mapping to the p-arm and 18q telomeric region confirmed comparative genomic hybridization (CGH)/spectral karyotyping (SKY) observations (data not shown).

In most cases, the cytogenetic analysis showed the whole 18q arm to be deleted, but there were exceptions. SW1417 was homozygous for microsatellites at *SMAD4* and showed loss of

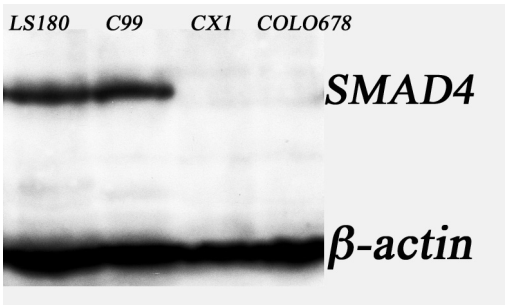

Fig. 1. Western blot analysis of SMAD4 and β-actin in colorectal cancer cell lines. Shown are the results of Western blot analysis using the B8 antibody against SMAD4, and a monoclonal anti-β-actin for four cell lines (LS180, C99, CX1, and COLO678). Note the complete absence of SMAD4 expression in CX1 and COLO678.

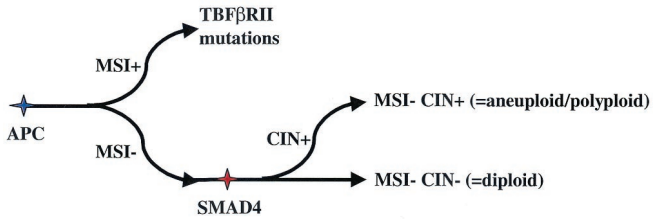

Fig. 3. Pathway showing possible sequence of events in tumorigenesis. Following mutations in the *APC* gene, a subset of tumors have inactivation of mismatch repair genes and so diverge along a pathway that includes mutations of the *TGFβRII* gene, and is characterized by microsatellite instability (MSI^+). A subset of the remaining tumors acquire mutations in the *SMAD4* gene, accompanied by loss of the wild-type chromosome 18, and this either precedes or causes another subset of tumors to diverge along a chromosomal instability (CIN^+) pathway with aneuploidy/polyploidy. The remaining tumors are MSI^-/CIN^-.

SMAD4 protein, but was heterozygous for markers near *DCC*. The microsatellite data for SW1417 were consistent with an interstitial deletion that targeted *SMAD4*, but left *DCC* intact. CGH data for SW1417 also showed a deletion around *SMAD4*, rather than loss of the whole arm. Conversely, C84 and SKC01 were heterozygous for microsatellites at *SMAD4*, but homozygous for microsatellites around *DCC*. Again this was substantiated by CGH data, which showed loss of chromosome 18 distal to 18q21. In these lines, SMAD4 expression was retained and it appeared that *DCC* or another tumor suppressor was being targeted, leaving *SMAD4* intact. The line HT29/CX1 had two apparently identical copies of chromosome 18 with *SMAD4* mutations, plus one deleted 18q. This line was heterozygous at D18S474, but homozygous at the rest of the microsatellites and for the *SMAD4* mutation, showing that the breakpoint for the deletion almost certainly lay just centromeric to *SMAD4*. Finally, cell line COLO678 was found to be homozygous for markers mapping to both the *SMAD4* and *DCC* regions, but heterozygous for markers on the p-arm and 18q telomeric region. Cytogenetic data were not available for this line, but presumably it contains an interstitial deletion targeting *SMAD4* and *DCC* without loss of the whole q-arm.

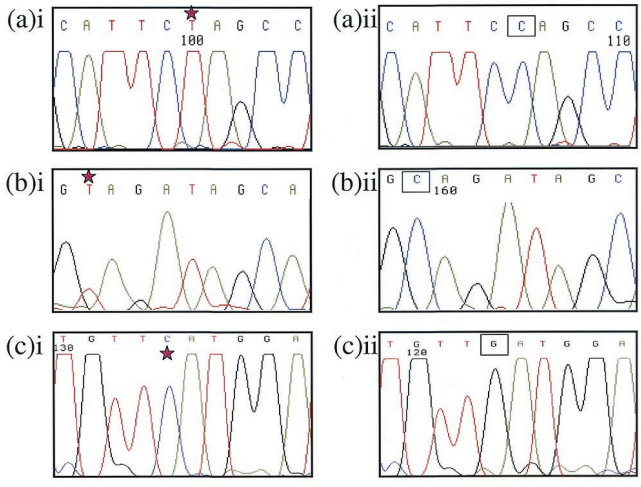

Fig. 2. Sequencing results of *SMAD4* for HT29/CX1, VACO10, and CACO2. *(a)i* shows the mutated cell line HT29/CX1 (c.931C>T transition causing Q311X amino acid truncation) compared with *(a)ii* wild type. *(b)i* SMAD4 exon 5 of VACO10 showing the c.715C>T transition causing Q239X truncation, compared with *(b)ii* wild type. *(c)i SMAD4* exon 8 of CACO2 showing the c.1051G>C nucleotide change, which results in a D351H missense change, compared with *(c)ii* wild type. Note the lack of a wild-type sequence underlying a mutated (starred) sequence. The corresponding wild-type base is shown in a box.

Discussion

This study aimed to clarify the role of *SMAD4* in the development of 44 colorectal cancer cell lines. Loss of SMAD4 expression and/or mutation were found in about half of the MSI^- lines, but in no MSI^+ lines. A broad spectrum of *SMAD4* mutations was seen; although, for unknown reasons, there were no frameshift changes. No association was made between the presence of a *SMAD4* mutation and tumor grade. Mutations appeared to occur more frequently in the C-terminal MH2 domain of *SMAD4*, although missense mutations in the N-terminal MH1 domain have been shown to cause *in vitro* protein instability (8, 24). Again, the reasons for this observation are unclear. In five of the fourteen CRC lines with absence of SMAD4 protein, no mutation was detected even after sequencing all exons in forward and reverse orientations, suggesting that cryptic *SMAD4* mutations or some other means of inactivating SMAD4 may have occurred. Inactivation of *SMAD4* due to hypermethylation has been shown not to occur (25) and, consistent with this finding, we detected *SMAD4* mRNA in PC/JW which had absent protein, but no detectable mutation. It is also possible that changes in a protein upstream of SMAD4 can sometimes lead to loss of SMAD4 expression.

In addition to mutations causing loss of protein, four cell lines with retained protein expression had missense *SMAD4* mutations in the MH2 region (Table 1). The missense mutations of C80 and CACO2 (D351H) occurred in the three loop–helix of the MH2 and have known pathogenic effects (26). The nearby R361C mutation, found not only in sporadic cancer, but also in the germline of JPS patients, is associated with undetectable levels of protein in JPS polyps, as assessed by using immunohistochemistry (data not shown). Mutations in the three-helix bundle of the MH2, such as R537Y (SW948) and L540R (SW1222), appear not to lead to unstable protein, but, like D351H, are predicted to abrogate protein function (26–28) by impairing the ability of SMAD4 to act as a homo- or hetero-oligomer and so interfering with signal transduction.

All lines with SMAD4 abnormalities were uniformly hemi- or homozygous at both *SMAD4* and *DCC* microsatellites and for the *SMAD4* mutation, showing that all copies of 18q were derived from the same chromosome 18 homologue, even where more than one copy of 18q21 was present in the cell. However, the dosage of 18q21 was always decreased relative to the overall level of ploidy. We found no cancer with more than one independent intragenic mutation in *SMAD4*. The simplest model to explain these data (Fig. 3) is one in which *SMAD4* mutations

occur before colorectal tumors acquire chromosomal instability (CIN) and polyploidy/aneuploidy, and are not late changes, as has been suggested (6, 8, 9). We believe that alternative explanations to this model are less plausible, because they would require several independent mutations to inactivate all copies of *SMAD4* and/or frequent, independent occurrence of identical *SMAD4* mutations in the same cell. However, Fodde *et al.* (29) have suggested that tetraploidy is a very early event in *APC*-mutant colorectal tumors, contrary to our favored model. Were the data of Fodde *et al.* (29) to be confirmed, we would be forced to conclude that some CRCs had lost three copies of 18q, acquired an intragenic *SMAD4* mutation, and reduplicated the remaining *SMAD4* mutant chromosome.

Given that none of 12 MSI^+ cell lines had *SMAD4* mutations or loss of *SMAD4* protein, it is most likely that the MSI^+ pathway had diverged before *SMAD4* inactivation occurred (Fig. 3). All eight MSI^+ lines previously studied have been reported to have *TGFBIIR* mutations (30, 31), whereas none of the ten MSI^- lines studied had *TGFBIIR* mutations (Fisher's exact test, $P < 0.000003$). Although inactivation of *SMAD4* and *TGFBIIR* may well not be functionally equivalent, these data certainly suggest that mutations of *TGFBIIR* occur after the divergence of the $MSI^{-/+}$ lineages. Thus, our model shows initial divergence of the MSI^+ pathway, followed by 18q loss and *SMAD4* mutation in the MSI^- cancers. Given that limited data suggest a high frequency of 18q loss in MSI^-CIN^- CRCs (32), divergence of the CIN^- and CIN^+ pathways probably occurs after 18q loss and *SMAD4* mutation.

Almost all MSI^- cell lines showed allelic loss, and even in MSI^- lines without defects in SMAD4, the dosage of 18q21 was always decreased relative to the overall level of ploidy: the question therefore remains as to the cause of 18q loss in lines with no evidence of SMAD4 inactivation. Although *SMAD2* remains an unlikely target, haplo-insufficiency of *SMAD4* is possible and *DCC* changes, although unlikely, cannot be excluded with certainty. Any other target genes on 18q must, like *SMAD4*, be altered after the MSI^+ pathway had diverged [although mutations in these gene(s) might, of course, be common to the MSI^+ and MSI^- pathways]. Given data which suggest that 18q21 loss occurs in colorectal adenomas or close to the adenoma–carcinoma transition (33), but that SMAD4 expression is lost only in CRCs (9), we speculate that 18q21 loss sometimes precedes mutation or silencing of the other copy of one or more target genes in the region.

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