Loss of Heterozygosity for Chromosome 18q and Microsatellite Instability Are Highly Consistent Across the Region of the *DCC* and *SMAD4* Genes in Colorectal Carcinomas and Adenomas

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ABSTRACT

Loss of heterozygosity (LOH) of chromosome 18q is clearly associated with colorectal carcinoma. The gene *DCC* (*Deleted in Colorectal Cancer*) is a putative tumor suppressor gene within the 18q21.2 region, but the importance of *DCC* has been questioned. A nearby gene called *SMAD4* has been suggested as the critical tumor suppressor gene in this region. We evaluated a series of 110 colorectal carcinomas and adenomas for LOH of the *DCC* and *SMAD4* genes to clarify the involvement of this DNA segment of chromosome 18q in colorectal carcinogenesis. We utilized 2 markers for each gene for all lesions and 5 additional markers for 23 lesions with inconsistent results, using standard PCR techniques. The benign and malignant portions of 50 *in situ* carcinomas were studied independently. Similar molecular results for LOH of *DCC* and *SMAD4* were found for 122 (79.7%) of 153 informative samples. Our results further show that the development of microsatellite instability within the q21-22 region of chromosome 18 clearly occurs prior to the histological change from adenoma to carcinoma, while LOH of this region of 18q is a late event in the neoplastic process. The physical proximity of these 2 genes will usually, but not invariably, result in similar LOH findings.

INTRODUCTION

Colorectal carcinoma is believed to develop through the accumulation of multiple mutations, either germ line and/or somatic, occurring in oncogenes, tumor suppressor genes, and DNA repair genes. Collectively, the genetic and epigenetic events endow the affected cells with self-sufficiency for growth.[1] Allelic loss, or loss of heterozygosity (LOH), involving several different chromosomal regions has been associated with colorectal carcinoma. One region shown repeatedly to experience LOH is

Table 1. Microsatellite Markers: Location and the Primers.

Marker	Locus	Forward primer listed first, reverse primer second for each marker
D18S45	18q11.1~11.2	5'- TTC TGG GTC ATG CAC ACA AT
		5'- GTA GTT ATT TTA AAG CCC GCC C
D18S1110	18q21.1	5'- GCA CGC AAT TCA AAA GCT AG
		5'- AAA GGC CTA GCA CCA CCT TAG
D18S474	18q21.1	5'- AGG CTG TCC TGT GCA CTA TG
		5'- TGT CAG AAG GCA TTT GTG ATG
D18S838	18q21.1	5'- AGC TTT ATC TTG AAG GTT GCA C
		5'- GTT TAC CCG GCC ATG AG
D18S1099	18q21.1	5'- CTA AAA GCG TCA CTC ACA CAG
		5'- TTA TAG CAA CAC AAG AAC AGC C
D18S1407	18q21.3	5'- TTC CCT TCA TTT CAC TGG GA
		5'- CTA GAT GGA TGT GAC TTG GC
D18S61	18q22.2	5'- ATT TCT AAG AGG ACT CCC AAA CT
		5'- ATA TTT TGA AAC TCA GGA GCA T
D18S58	18q22.3	5'- GCT CCC GGC TGG TTT T
		5'- GCA GGA AAT CGC AGG AAC TT
D18S70	18q23.0	5'- AAG GCT GAN CTC TAC CG
		5'- GGA ATG TCA AGA AGT ACC TAC CAT A

18q.[2] A putative tumor suppressor gene was identified within the 18q21.2 region and is referred to as *DCC* (*Deleted in Colorectal Cancer*).[3]

However, much of the reported data on loss and inactivation of the *DCC* gene is circumstantial and fails to provide conclusive evidence that the *DCC* gene functions as a tumor suppressor gene, rather than merely being an epiphenomenon.[4] These authors point out there is no evidence that germ line mutations of the *DCC* gene play a role in heritable cancer, unlike true tumor suppressor genes. Further, very few somatic mutations in the *DCC* gene have been reported in colorectal carcinoma tissue.[4] With other tumor suppressor genes, somatic mutations are frequently responsible for the inactivation of the remaining allele, following LOH of one of the gene pair. Another gene, referred to as *SMAD4*, also found in the region affected by 18q LOH, has been suggested as the critical putative tumor suppressor gene in colorectal carcinomas.[4] The *SMAD4* gene encodes a protein involved with transforming growth factor beta (TGF-beta) signaling. Transforming growth factor beta normally has inhibitory effects on colonic epithelial cells, and loss of TGF-beta function, as a result of loss of *SMAD4* function could, therefore, be important in colorectal carcinoma development.[5,6]

We evaluated a series of colorectal carcinomas and adenomas for LOH of the *DCC* and *SMAD4* genes to better understand the involvement of this DNA segment of chromosome 18q in colorectal carcinogenesis. Two markers frequently used to assess for LOH for each gene were initially assessed. When LOH results were discrepant with this first set, additional markers were then utilized. A series of *in situ* carcinomas (ISCs) was also studied by separately assaying the benign adenomatous portion as well as the malignant portion to compare the frequency of LOH of this chromosomal region in the 2 stages of the evolving neoplasm. Each area of the *in situ* neoplasms could be assessed independently, since our focus is the comparison of markers to each other. Finally, DNA from a series of samples

Figure 1. Ideogram of 18q showing the loci of microsatellite markers and the *SMAD4* and *DCC* genes.

was sequenced to assess the major hot spot for mutations within the *SMAD4* gene.

MATERIALS AND METHODS
LOH Determinations

Fifty percent of 220 tissue DNA samples of neoplasms from 2 prior molecular pathology studies were randomly picked for this analysis.[7,8] Paraffin blocks were available from storage. No protected health information was sought for any patient, thus the protocol was approved by the hospital Institutional Review Board with a Health Insurance Portability and Accountability Act (HIPAA) waiver.

Loss of heterozygosity of the *SMAD4* gene region was initially determined by polymerase chain reaction (PCR) amplification of the microsatellite CA repeats D18S474 and D18S1110. Loss of heterozygosity of the *DCC* gene region was determined by amplification of the CA repeat markers within the D18S58 and D18S61 loci. Additional markers utilized included D18S45, D18S838, D18S1099, D18S1407, and D18S70. Primer sets were abstracted from information listed on the National Center for Biotechnology Information (NCBI) Web site (www.ncbi.nlm.nih.gov) or the Ensembl Web site (www.ensembl.org). The primer sets used are showed in Table 1, and the relative locations of all markers are shown in Figure 1. Labeled primers were obtained from the Applied Biosystems Custom Oligo Synthesis Service (OligosUS@appliedbiosystems.com). In the primer sets, 1 primer contained a 5' fluorescent label while the other primer contained a 5'-GTGTCTT tail.

Polymerase chain reactions were carried out in 30 μL volumes using Applied Biosystems reagents (Roche Molecular Systems, Inc., Branchburg, NJ). Four picomoles of each primer and a 1.5-mM $MgCl_2$ concentration were used in the
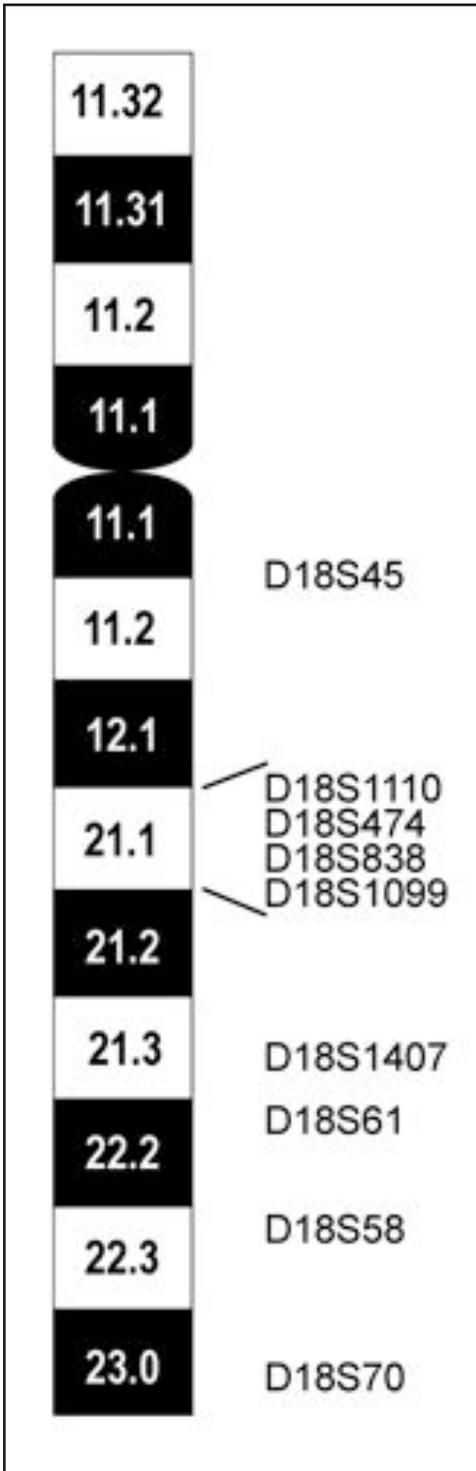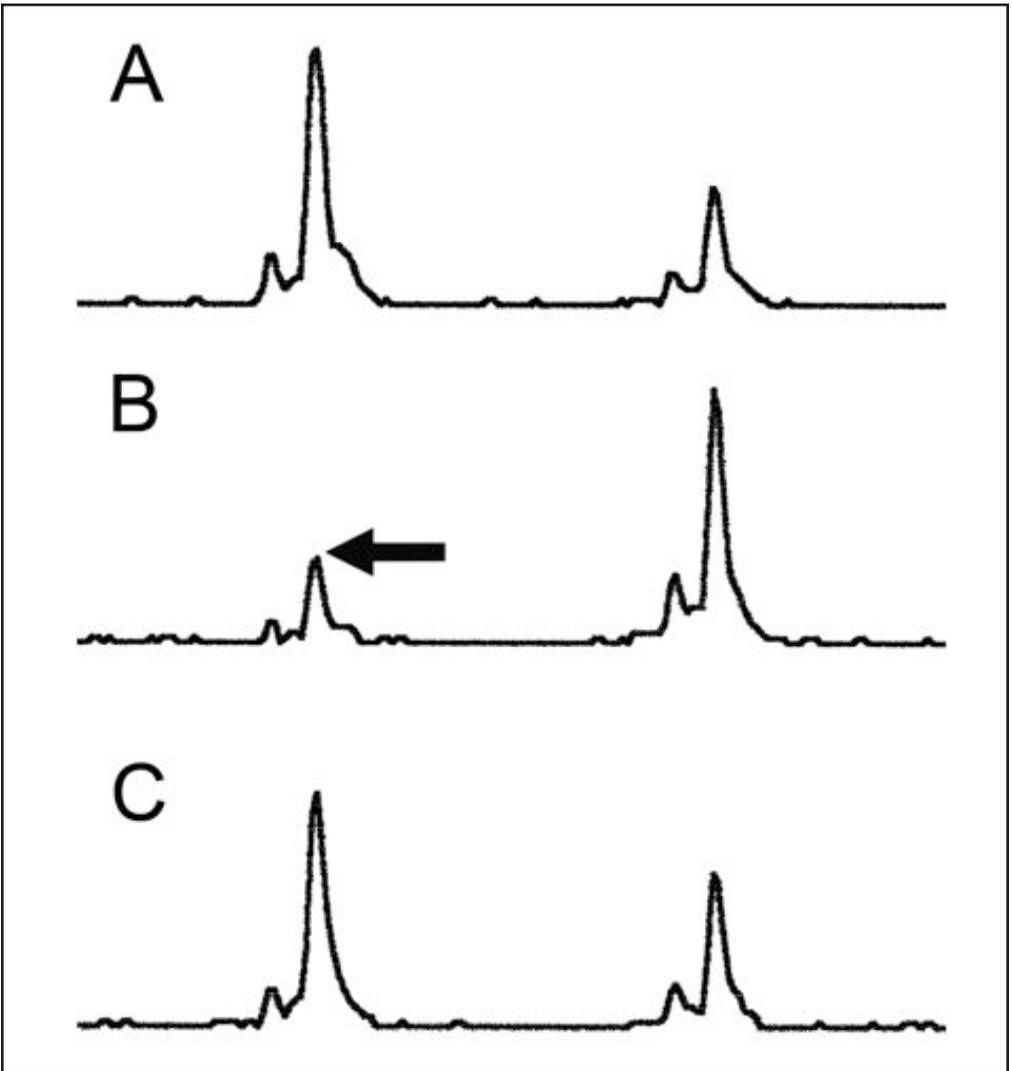
Figure 2. A sample plot or electropherogram showing D18S1110 microsatellite analysis of DNA used to determine LOH. A. DNA from normal colon mucosa indicating the patient's normal 2 alleles. B. DNA from the carcinomatous portion of an *in situ* lesion from the ascending colon. There is significant loss of the intensity of the left peak indicating LOH. C. DNA from the villous adenomatous portion of the lesion. The relative heights of the 2 peaks are very close to the ratio of the respective peaks for the normal tissue, indicating no LOH (normalized ratio = 0.73).

reactions. Reactions were run on a PE 9700 thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: 6 minutes denaturation at 94°C, followed by 35 cycles of a 30-second denaturation at 94°C, 25-second annealing at 55°C, and a 50-second elongation at 72°C, with a final 30-minute extension at 72°C. Polymerase chain reactions products were analyzed on an ABI 3130 Genetic Analyzer with GeneMapper software (Applied Biosystems, Foster City, CA). For all LOH studies, neoplastic tissue was evaluated simultaneously with normal colonic mucosal tissue from the same patient. The ratio of the height of the allele band intensities of the neoplastic tissue was divided by the corresponding ratio for the normal tissue, and LOH was
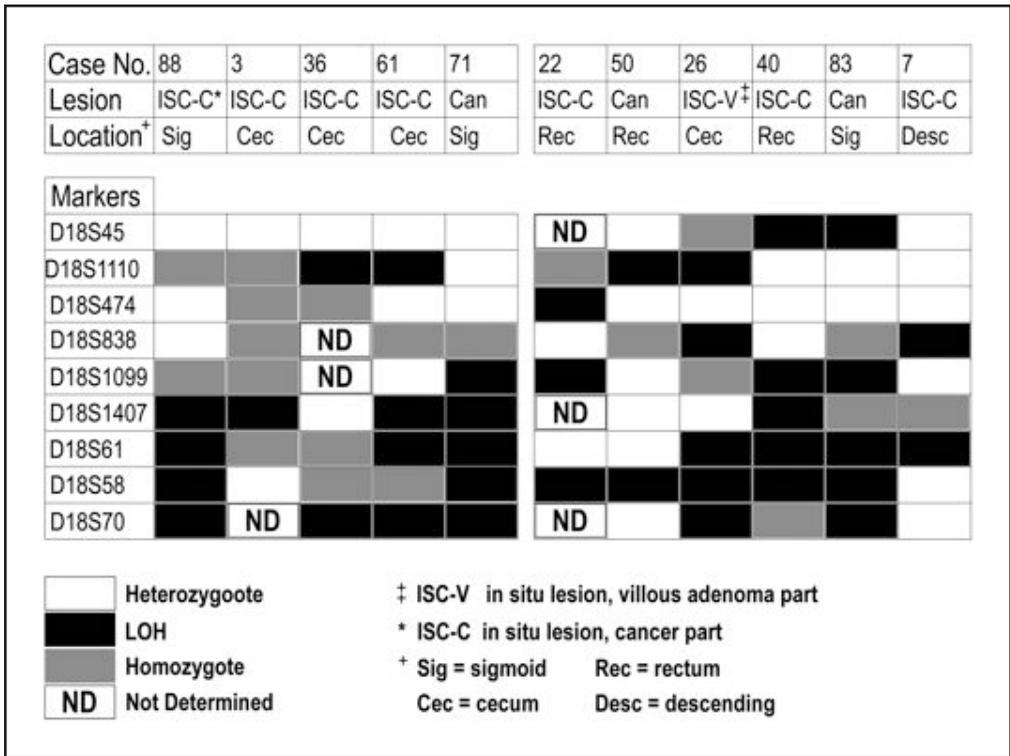
Figure 3. Deletion map of patients with colorectal neoplasms. A. Five samples with LOH primarily or exclusively demonstrated with telomeric markers. B. Six samples with more variable distribution of LOH.

Case No.	88	3	36	61	71		22	50	26	40	83	7
Lesion	ISC-C*	ISC-C	ISC-C	ISC-C	Can		ISC-C	Can	ISC-V‡	ISC-C	Can	ISC-C
Location+	Sig	Cec	Cec	Cec	Sig		Rec	Rec	Cec	Rec	Sig	Desc

Markers												
D18S45							ND					
D18S1110												
D18S474												
D18S838			ND									
D18S1099			ND									
D18S1407							ND					
D18S61												
D18S58												
D18S70		ND					ND					

Legend:
- Heterozygote
- LOH
- Homozygote
- ND — Not Determined
- ‡ ISC-V in situ lesion, villous adenoma part
- * ISC-C in situ lesion, cancer part
- + Sig = sigmoid   Rec = rectum
- Cec = cecum   Desc = descending

defined as a resultant ratio of ≤0.5.[9] A representative plot is shown in Figure 2.

Microsatellite Instability (MSI) Testing
All lesions had previously been assayed for *APC* gene LOH using marker D5S346, part of the Bethesda panel for MSI analysis. Any sample showing MSI with the four 18q markers was additionally assayed with the marker BAT26. Microsatellite instability by the 3 markers was considered MSI-high.

***SMAD4* Gene Sequencing**
Exon 8 of the *SMAD4* gene was sequenced to detect somatic mutations within this region. Primers were designed from sequence information listed at the NCBI Web site under accession AF045438. Primers were ordered through Sigma Genosys. (www.sigma-genosys.com). The primer set is 5'- GAT GTT CTT TCC CAT TTA TTT CC-3' (sense) and 5'- TAA AGT AAC TAT CTG ACT ATA CAA TC-3' (antisense), and it generates a 243-base pair PCR product spanning codons 319 to 379. Hot-start PCR reactions were run on a PE 9700 themocycler (Applied Biosystems, Foster City, CA) using 75 picomoles of each primer and a 1.75-mM $MgCl_2$ concentration. The reaction tubes were initially denatured for 6 minutes at 94°C, cooled to 90°C and paused to add Taq polymerase, and then heated for an additional 1 minute at 94°C. This was followed by 35 cycles of a 30-second denaturation at 94°C, 25-second annealing at 50°C, and a 50-second elongation at 72°C, with a final 5-minute extension at 72°C. Post PCR product was purified and sequenced, and then separated on a

Table 2. Results of Assays for LOH at 4 Markers for 50 *in situ* Colorectal Carcinomas.

Marker: Adenomatous Portion/ CarcinomatousPortion	D18S58	D18S61	D18S474	D18S1110
MSI/MSI	5	5	6	7
LOH/LOH	6	5	3	7
Normal/LOH	7	15	12	15
LOH/Normal	0	3	2	2
Normal/MSI	0	1	0	0
Normal/Normal	7	15	16	12
Homozygous	15	5	11	4
Not assayed	10	1	0	3
LOH = loss of heterozygosity; MSI = microsatellite instability.				

5% Long Ranger acrylamide gel (Cambrex, Rockland, ME).

RESULTS

The 110 lesions were removed from 88 individuals, 47 females and 41 males. The average age at the time of removal was 71.2 years. Fifty of the 110 lesions were ISCs that could be easily separated into benign and malignant parts, yielding an additional 50 samples for analysis. The lesions studied were: carcinomas, 42; ISCs, 50, (44 had residual villous adenoma and 6 had residual tubular adenoma); villous and tubulovillous adenomas, 9; tubular adenomas, 8; and hyperplastic polyp, 1. Lesions were from all segments of the colon: cecum, 22; ascending, 28; transverse, 13; descending, 5; sigmoid, 24; and rectum, 18. Seven carcinomas from 7 different patients revealed MSI by all 4 initial markers. These lesions also demonstrated MSI with the markers D5S346 and BAT26, indicating MSI-high. No other lesions demonstrated MSI-high. Of these 7 patients, 6 were female and 1 was male. Their average age at the time of diagnosis was 78.1 years. Six of the 7 MSI-positive lesions were from the right colon and 1 was from the sigmoid. The prevalence of homozygosity for the 4 primary markers used was as follows: 25 of 78 (32%) individuals were homozygous for D18S58 and 12 of 78 (15%) were homozygous

for D18S61; 16 of 79 (20.3%) individuals were homozygous for D18S474 and 5 of 79 (6.3%) for D18S1110.

Comparison of 4 Initial Markers
The combined results of the 2 *DCC* markers (D18S58 and D18S61) were compared to the combined results for the 2 *SMAD4* markers (D18S474 and D18S1110). The 160 separate samples yielded 7 samples from patients homozygous for 3 of the 4 markers. Five of these samples yielded consistent results between the informative, fourth primary marker, and 3 additional markers (4 lesions showed no LOH and 1 lesion showed LOH), but 2 samples revealed discrepant results and were then studied further with the complete expanded panel.

Results for the remaining 153 samples were considered consistent if results from all 4 markers demonstrated the same result, or if at least one of each pair of markers demonstrated the same result, with the other marker either homozygous or not studied. There were 122 (79.7%) samples with the same results: 66 (54.1%) normal, 44 (36.1%) with LOH, and 12 (9.8%) samples with MSI. There were 31 (20.3%) samples with discrepant results, and DNA was available for study with the expanded panel for 21 of these samples.

Thus, 23 samples were further stud-

ied using 5 additional markers for a total of 9 markers. The results could be separated into 4 groups: A. Eight samples gave consistent results save for 1 marker; 5 were predominately LOH and 3 were predominately normal. B. Four samples, representing the 2 parts of 2 *in situ* lesions, revealed a mix of MSI and normal, but with MSI seen across the region. These 2 *in situ* neoplasms did not demonstrate MSI with markers D5S346 or BAT26. C. Five samples revealed little or no LOH with centromeric markers and LOH with telomeric markers. D. Results for 6 samples were more variable. (Groups C & D are illustrated in Figure 3.)

Comparison of the 2 Histologically Different Areas of *in situ* Lesions

Fifty ISCs were studied with the 4 markers D18S58, D18S61, D18S474, and D18S1110. Five ISCs were informative for just 1 marker. A total of 45 ISCs were informative for both the benign and malignant portions for at least 2 of the 4 markers. For 32 of these ISCs (71%), the marker results were consistent: adenoma/cancer both normal, 12; both LOH, 2; both MSI, 5; normal/LOH, 12, and LOH/normal, 1. Of the 13 ISCs with inconsistent results among the four markers, 10 varied by just 1 marker (Table 2).

Sequencing of *SMAD4* Gene

Twenty four lesions showing LOH with both D18S474 and D18S1110, or for one marker with the other marker homozygous, were sequenced looking for possible mutations in exon 8, codons 319 to 379. Twenty three of the 24 lesions were carcinomas, and 1 lesion was a tubular adenoma. All 24 lesions revealed no abnormalities in the exon 8 region by sequencing.

DISCUSSION

Multiple genetic alterations are involved in the initiation and progression of carcinogenesis. These affect both proto-oncogenes and tumor suppressor genes. Two "hits" are needed for inactivation of tumor suppressor genes; the first is often a point mutation, either germ line or somatic. The second "hit" is the elimination of the wild-type allele through nondisjunction, deletion, recombination, and/or chromosome loss and duplication. This results in the primary mutation becoming homozygous or hemizygous, thus the term "loss of heterozygosity."[10]

Loss of heterozygosity of chromosome 18q is common in primary colorectal carcinoma, but it is infrequent in small adenomas, leading to the conclusion that 18q LOH may contribute more to progression rather than initiation of colorectal carcinoma. More than 90% of primary colorectal carcinomas with LOH of chromosome 18q have allelic loss of the region that includes the *DCC* gene.[11] The *DCC* gene is quite large, with 29 or more exons, and it spans a large genomic region of about 1.2 million base pairs.[12] The *DCC* gene encodes several different protein products as a result of alternative splicing. All known isoforms appear to be transmembrane glycoproteins. The extracellular domain is similar to that found in the neural cell adhesion molecule protein family.[4] The expression of DCC protein, as determined by immunohistochemistry, has been reported as a strong positive predictive factor for survival in stage II and III colorectal carcinomas.[13] However, in a mouse model, inactivation of the murine *DCC* gene did not affect growth, differentiation, morphogenesis, or tumorigenesis in the mouse intestine.[14]

The *SMAD4* gene was first identified as a tumor suppressor gene of pancreatic cancer in 1996 and was designated as *DPC4* (*deleted in pancreatic carcinoma, locus 4*).[15] The human *DPC4* gene contains 11 exons with a predicted 155

amino acid coding sequence, or just 466 base pairs.[15] The DPC4 protein sequence has similarities to the *Drosophila melanogster* Mad (mothers against dpp) protein, and to the *Caenorhabditis elegans* Mad homologs sma-2, sma-3 and sma-4.[16] The *DPC4* gene is the human homolog of sma-4 and is referred to as *SMAD4*.

The *SMAD4* gene, located at 18q21.1, is closer to the centromere of chromosome 18 than is the *DCC* gene. Germ line mutations in the *SMAD4* gene have been shown to be associated with juvenile polyposis, an autosomal dominant syndrome predisposing to hamartomatous polyps and colorectal carcinoma.[17] Animal studies have shown that *SMAD4* gene inactivation is involved in the malignant transformation of gastrointestinal adenomas.[18] Loss of *SMAD4* gene expression has been shown to be highly correlated with the loss of expression of E-cadherin, which is important in the control of cell adhesion.[19] These relationships indirectly support *SMAD4*, rather than *DCC*, as the critical gene in the 18q region.

We found the marker D18S61 to be more informative than D18S58, as it was twice as likely to be heterozygous. D18S1110 was a more informative marker than D18S474, as it was 3 times more likely to be heterozygous. Results among the 4 primary markers were consistent in 80% of samples.

The results obtained by evaluating 23 samples with 5 additional markers indicated several interesting points. First, homozygosity may not be consistent across this region of chromosome 18. As indicated in Figure 3, regions of homozygosity may be interspersed with heterozygous regions. Second, MSI may not be appreciated with all markers. We believe this results from technical considerations, including the sensitivity of the various markers for detecting extra peaks. Third, for a minority of samples,

one marker may fail to show LOH, while all other markers for the region suggest LOH. We found the markers D18S110 and D18S61 to be least reliable in this regard, probably for technical reasons. For example, some markers provide a better separation of the 2 alleles than others, thereby facilitating the interpretation of LOH. Fourth, with 5 of our samples, the ratios for the informative centromeric markers were around 1.0, while the telomeric markers gave ratios well below 0.5. This would suggest that in a small minority of neoplasms, LOH might be present in the more distal area of this chromosomal region but not in the more proximal, or centromeric, area.

Sequencing the *SMAD4* gene has been reported for a total of 353 carcinomas, with mutations detected in 49 (13.9%).[20-26] The mutation was reported to be in exon 8, between codons 319 and 379, for 17 of the 49 (34.7%) carcinomas with a documented mutation, but in exon 8 for just 4.8% of all carcinomas (17 of 353). We found no sequencing abnormalities in exon 8 for the 24 lesions studied. However, based upon the 4.8% rate for mutations in exon 8, we would have anticipated finding only 1 lesion with an exon 8 mutation (0.048 × 24).

The development of MSI for the q21-22 region of chromosome 18 clearly occurs prior to the histological change from adenoma to carcinoma, as all 7 *in situ* lesions demonstrating MSI in the carcinomatous portion also showed MSI in the adenomatous portion. This was demonstrated equally well with all markers used. Furthermore, when MSI occurs, it is clearly present across the entire region studied.

There are few literature reports of *SMAD4* LOH or mutations in adenomas. Miyaki et al[23] reported no *SMAD4* mutations detected in 40 adenomas but LOH was demonstrated for 1 adenoma.

Maitra and colleagues[27] reported that 19 adenomas evaluated by immunohisto-chemistry for SMAD4 protein were all positive, suggesting normal expression of the gene. We studied 17 independent adenomas and just 1, a sigmoid tubulovillous adenoma, showed LOH for 2 of the initial 4 markers. For several of the *in situ* lesions with LOH in the carcinomatous portion, the adenomatous portion also demonstrated LOH with each marker used. However, for the majority of ISCs where the carcinomatous portion demonstrated LOH, the residual adenomatous area of the lesion did not. This supports the concept that LOH of this region of chromosome 18 is frequently a late event in the neoplastic process. Precisely when these molecular changes occur may not be identical in all cases, but may reflect different genetic predispositions and different mutagenic influences to which patients are exposed. Furthermore, there were 4 different *in situ* lesions with LOH demonstrated in the adenomatous portion but not in the carcinomatous portion, for 1 or more marker. This supports the concept that initial molecular genetic changes in an adenoma are not always carried along into the developing carcinomatous lesion, a process that has been suggested by others.[28]

In summary, similar molecular results for markers in the region of chromosome 18 containing the genes *DCC* and *SMAD4* were found for 122 (79.7%) of 153 of our informative samples. This indicates that the physical proximity of these 2 genes will usually, but not invariably, result in their sharing a similar molecular fate. Our data do not permit a conclusion regarding whether *DCC* or *SMAD4* is the more biologically relevant gene involved in the neoplastic process, to the exclusion of the other. It is possible that one of the 2 genes may be more critical than the other for certain lesions at particular stages of neoplasm development.

Our data show that the development of MSI occurs prior to the histological change from adenoma to carcinoma, while LOH of this region is frequently a late event in the neoplastic process.

CONFLICT OF INTEREST STATEMENT
All authors report they have no conflict of interest with respect to this study.

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