Loss of Smad Signaling in Human Colorectal Cancer Is Associated with Advanced Disease and Poor Prognosis

Wen Xie, PhD,[a] *New Brunswick, New Jersey*, David L. Rimm, MD, PhD,[c] *New Haven, Connecticut*, Yong Lin, PhD,[b] Weichung J. Shih, PhD,[b] Michael Reiss, MD,[a] *New Brunswick, New Jersey*

PURPOSE

Based largely on in vitro investigations and animal studies, investigators believe that disruptions of transforming growth factor-β (TGF-β) signaling contribute to the development and progression of human colorectal cancer. The purpose of this study was to directly assess the status of the TGF-β signaling pathway in colorectal cancer and determine the effects of its disruption on clinical behavior and outcome.

MATERIALS AND METHODS

Smad proteins are the principal intracellular components of the TGF-β signaling pathway. We conducted a high-throughput analysis of the expression patterns of Smad2, phosphorylated (activated) Smad2 (pSmad2), and Smad4 in more than 600 human colorectal cancer specimens assembled in tissue microarrays.

RESULTS

The vast majority (93.8%; 95% CI: 92%–96%) of colorectal cancers expressed phosphorylated Smad2, indicating the ability of the tumors to survive and proliferate within a microenvironment that contains bioactive TGF-β. Twelve of 633 (1.9%; 95% CI: 1%–3%) cases failed to express Smad2, and 15 of 641 (2.3%; 95% CI: 1%–4%) cases failed to express Smad4. Moreover, 29 of 615 (4.7%; 95% CI: 3%–7%) of cases expressed Smad2 but not its activated form (pSmad2), suggesting the presence of a TGF-β receptor defect. Based on an analysis of 577 cases for which clinical outcome information was available, failure to express Smad2, pSmad2, or Smad4 was associated with advanced-stage disease, the presence of lymph node metastases, and a significantly shorter overall survival (median survival: 35 vs 58 months).

DISCUSSION

Loss of Smad activation and/or expression occurs in approximately 10% of colorectal cancers. This subset has a poor prognosis because of its association with advanced disease and the presence of lymph node metastases at diagnosis. *(Cancer J 2003;9:302–312)*

KEY WORDS:
Transforming growth factor-β, Smad, prognosis, colorectal cancer

The transforming growth factor-β (TGF-β) superfamily of secreted polypeptides regulates cell proliferation, differentiation, motility, and apoptosis in different cell types, including intestinal epithelial cells.[1,2] The TGF-β signal is transduced by a pair of transmembrane serine-threonine kinase receptors.[2] Binding of TGF-β to type II receptor (TβR-II) homodimers results in the recruitment of two type I receptor (TβR-I) molecules into heterotetrameric complexes, which in turn results in activation of the TβR-I kinase by TβR-II. In response to receptor activation, two cytosolic proteins, Smad2 and Smad3, become transiently associated with and phosphorylated by the TβR-I kinase, allowing them to form heteromeric complexes with a third homologue, Smad4. These complexes are translocated to the nucleus, bind to DNA in a sequence-specific manner, and regulate gene transcription.[2] In untransformed intestinal epithelial cells, TGF-β1 potently inhibits cell cycle progression,[3,4] primarily by repressing cyclin D1 and cdk4 expression.[5-7] In addition, TGF-β1 induces differentiation of intestinal epithelial cells and appears to promote mucosal healing by enhancing epithelial cell motility and migration.[3,8-10]

Besides these physiologic functions of TGF-β, there is considerable evidence that alterations of TGF-β/Smad signaling play an important role in colon carcinogene-

From the [a]Division of Medical Oncology, Department of Internal Medicine, the [b]Division of Biometrics, The Cancer Institute of New Jersey, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, New Jersey, and the [c]Department of Pathology, Yale Cancer Center, Yale University School of Medicine, New Haven, Connecticut.

Received on January 30, 2003; accepted for publication April 16, 2003.

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this article.

Reprint requests: Michael Reiss, MD, Division of Medical Oncology, Department of Internal Medicine, UMDNJ-Robert Wood Johnson Medical School and The Cancer Institute of New Jersey, Room 2007, 195 Little Albany Street, New Brunswick, NJ 08903. E-mail: michael.reiss@umdnj.edu.

sis.[11] For example, one strain of Smad3 mutant mice develops highly invasive and metastatic colorectal adenocarcinomas between 4 and 6 months of age.[12] Furthermore, in compound heterozygote mice that carry both adenomatous polyposis coli (*APC*) and *Smad4* gene mutations, intestinal polyps develop into malignant tumors at an accelerated rate compared to the simple *APC* mutant heterozygotes.[13] Moreover, *Smad4* heterozygous mice eventually develop intestinal polyps and invasive carcinomas at 6–12 months of age.[14,15] In these cases, loss of the second, wild-type *Smad4* allele occurs at a late stage of tumor development.[15] Thus, haploid insufficiency of *Smad4* appears to support tumor initiation, whereas biallelic loss contributes to later stages of tumor progression. In man, germline mutations of either the TβR-II receptor or Smad4 confer a high risk of developing gastrointestinal cancers.[16,17]

Secondly, numerous in vitro studies have demonstrated that transformed colon epithelial cells progressively loose the growth inhibitory response to TGF-β in parallel with the stage of the tumor of origin.[11] Moreover, TGF-β–overexpressing colon tumors may represent a particularly aggressive subset because the prognosis of patients whose cancers overexpress TGF-β appears to be worse than that of nonexpressors.[18]

Although molecular genetic studies of colorectal cancer have identified two main subtypes with different underlying forms of genetic instability, the TGF-β signaling pathway appears to be affected in both types.[19] Most colorectal cancers are characterized by allelic losses involving chromosome 18q, which contains the Smad2 and Smad4 gene loci. Approximately 10% and 20% of sporadic colorectal carcinomas have been estimated to carry mutations in the *Smad2* and *Smad4* genes, respectively.[20-25] Loss of expression of Smad4 protein was noted in two of 14 cases (14%) of colorectal cancer.[26] Conversely, between 70% and 90% of colorectal carcinomas and carcinoma cell lines associated with microsatellite instability due to hereditary or acquired DNA mismatch repair deficiencies (the so-called RER + phenotype) display intragenic mutations of *TβR-II* that result in loss of its tumor suppressive activity.[27-39] In addition, missense mutations in the *TβR-II* gene have been reported to occur in approximately 15% of microsatellite-stable colorectal cancer cell lines.[40]

To address the questions of whether and how disruptions of TGF-β/Smad signaling affect outcome of patients with colorectal cancer in more detail, we have conducted a retrospective high-throughput tissue microarray analysis of a large cohort of unselected colorectal cancers. In this study, we used an immunohistochemical approach that allowed us to examine the functional status of the TGF-β receptors as well as receptor-associated and common Smads. Our findings indicate that inactivation of any of these elements of the TGF-β signaling pathway occurs in approximately 10% of colorectal cancers and is associated with advanced disease and, consequently, with a poor prognosis.

MATERIALS AND METHODS

Construction and Processing of Colon Cancer Tissue Microarrays

Tissue microarrays were assembled using formalin-fixed, paraffin-embedded tissue blocks retrieved from the archives of the Yale University School of Medicine Department of Pathology, as previously described.[41] Each tissue specimen was represented in the arrays by at least two cores. Two separate tissue microarrays were used in this study. The first array contained 45 cases of primary invasive colorectal cancer diagnosed in 1999. The second and largest array contained 650 cases of primary invasive colorectal cancers, each represented by two cores. For 624 of these cases, follow-up information was available (median follow-up: 53 months). This study was approved by the institutional review board.

Detection of Smad Proteins Using Immunohistochemistry

Smad proteins were identified in 5-μm tissue microarray sections by use of a polyclonal goat anti-Smad2 (S-20) antibody (1:100; Santa Cruz Inc., CA), a polyclonal rabbit anti-phospho-Smad2 (1:100; pSmad2) antibody[41-43]), or a monoclonal mouse anti-Smad4 (B-8) antibody (1:150; Santa Cruz Inc., CA), as previously described.[41] The intensity of positive staining was scored independently by two observers.[41,43] Cases of disagreement were re-reviewed jointly to arrive at a consensus score. Disk scores from the same tumor were averaged to produce a single score.[44]

Statistical Analysis

Survival curves were estimated according to the Kaplan-Meier method.[45] For each curve, the starting point was the date of diagnosis of colon cancer. Death from any cause was counted as an event in the calculation of overall survival time. For surviving patients, time was censored at the last available follow-up date. The median follow-up time in this series was 53 months. The log-rank test (Mantel-Cox) was used to compare outcomes of different groups. Proportional-hazards regression models were used for multivariable comparisons of time-to-event endpoints.[46] Contingency table analyses using the Fisher's exact test were used to determine the relationships between Smad status and known prognostic factors for colorectal carcinoma. All analyses were performed using Abacus Concepts, Statview 4.51 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Smad Expression Patterns in Normal Colon Tissue

We first examined the pattern of Smad expression and activation in normal colonic epithelium (Fig. 1). As expected, all normal tissue elements, including epithelial and stromal cells, as well as capillaries, expressed Smad2 as well as Smad4. The staining was predominantly cytoplasmic in the case of Smad2 and mixed cytoplasmic and nuclear in the case of Smad4. However, to our surprise, the activated form of Smad2 (pSmad2) was clearly detectable in the cytoplasm and nuclei of epithelial cells and the stromal elements of normal crypts of Lieberkühn, as well as in the capillary endothelial cells (Fig. 1). Furthermore, the intensity of pSmad2 staining of the epithelial and the stromal cells seemed to increase along the crypt axis, from relatively weak at the base of the crypts to stronger toward the luminal surface. These findings suggest that biologically active TGF-β is present within the microenvironment immediately surrounding normal colonic crypts and capillaries and that a relationship may exist between Smad2 activation and the differentiation of the epithelial cells as they move up toward the luminal surface.

Smad Expression Patterns in Colon Cancer Tissue Microarrays

In order to determine the status of Smad signaling in invasive human colon carcinomas in vivo, Smad2, pSmad2, and Smad4 expression were examined by immunostaining of tissue microarrays (Table 1). The two microarrays included a total of 695 primary invasive carcinomas of the colon or rectum. Of 633 evaluable cases, 621 (98.1%; 95% CI: 97%–99%) expressed Smad2 (Table 1). The remaining 12 (2%; 95% CI: 1%–3%) cases failed to express Smad2 protein, probably reflecting inactivation of the Smad2 gene by deletion or intragenic mutation.[23] Phosphorylation of receptor-activated Smads by ligand-induced activation of the TGF-β receptor complex is a key step in the intracellular transduction of TGF-β signaling. Our anti-pSmad2 antibody allowed us to assess the state of activation of receptor-associated Smad2 by the TβR receptor complex in tumor tissue in situ.[41] Diffuse positive cytoplasmic as well as nuclear staining for pSmad2 occurred in 615 (93.8%; 95% CI: 92%–95%) of 656 colorectal cancer specimens (Table 1, Figs. 1 and 2). This is an important finding because it indicates that TGF-β receptor signaling is activated in the vast majority of invasive colorectal carcinomas. By inference, then, these tumor cells are capable of proliferation in spite of an activated TGF-β signaling pathway. This finding indicates not only that biologically active TGF-β is present within the microenvironment of these tumors but also that the carcinoma cells must have escaped from TGF-β–mediated cell cycle arrest. In the 41 remaining cases (6.3%; 95% CI: 5%–8%), we were unable to detect pSmad2 expression within the tumor cells, although it was present in surrounding stromal cells and capillaries (Fig. 2). Thus, these cases are likely to have lost expression or to have acquired inactivating mutations of one or the other TGF-β receptor subtype.[27,40]

Smad4 expression was observed in 626 (98%; 95% CI: 96%–99%) of 641 evaluable colorectal cancer specimens (Table 1, Fig. 2). Positive staining was predominantly confined to the cytoplasm of tumor epithelial cells, with occasional associated nuclear staining. Smad4 was undetectable in the tumor cells in the remaining 15 cases (2.3%; 95% CI: 1%–4%). Recent studies have demonstrated that loss of immunostainable Smad4 protein as assayed by use of the B-8 monoclonal antibody (Santa Cruz) is an extremely sensitive and specific surrogate marker for structural alterations of the Smad4 gene in tumor specimens.[47,48] Thus, it is likely that the loss of Smad4 expression in our series is also the result of either loss or mutation of both alleles of the Smad4 gene.

In summary, based on the expression pattern of Smad2, pSmad2, and Smad4, colorectal cancers displayed one of six different phenotypes (Table 2): most cases ($N = 554$) co-expressed Smad2, pSmad2, and Smad4. Among the remaining 32 cases, seven were Smad2 negative, 14 were pSmad2 negative, four were Smad4 negative, and seven had a dual defect in TGF-β signaling (Smad2 and Smad4 negative: four cases; pSmad2 and Smad4 negative: three cases).

Associations Between Losses of Smad Signaling and Clinical Outcome

Numerous small studies have suggested that alterations of TGF-β signaling in colorectal cancers might have

TABLE 1 Smad Expression and Activation in Colorectal Cancer Tissue Microarrays: Classification by Individual Smad[a]

Smad2		pSmad2		Smad4	
Positive	Negative	Positive	Negative	Positive	Negative
621 (98.1%)	12 (1.9%)	615 (93.8%)	41 (6.2%)	626 (97.7%)	15 (2.3%)

[a]Total numbers of cases with positive versus negative Smad immunostaining in colorectal cancer tissue microarrays (see Materials and Methods).

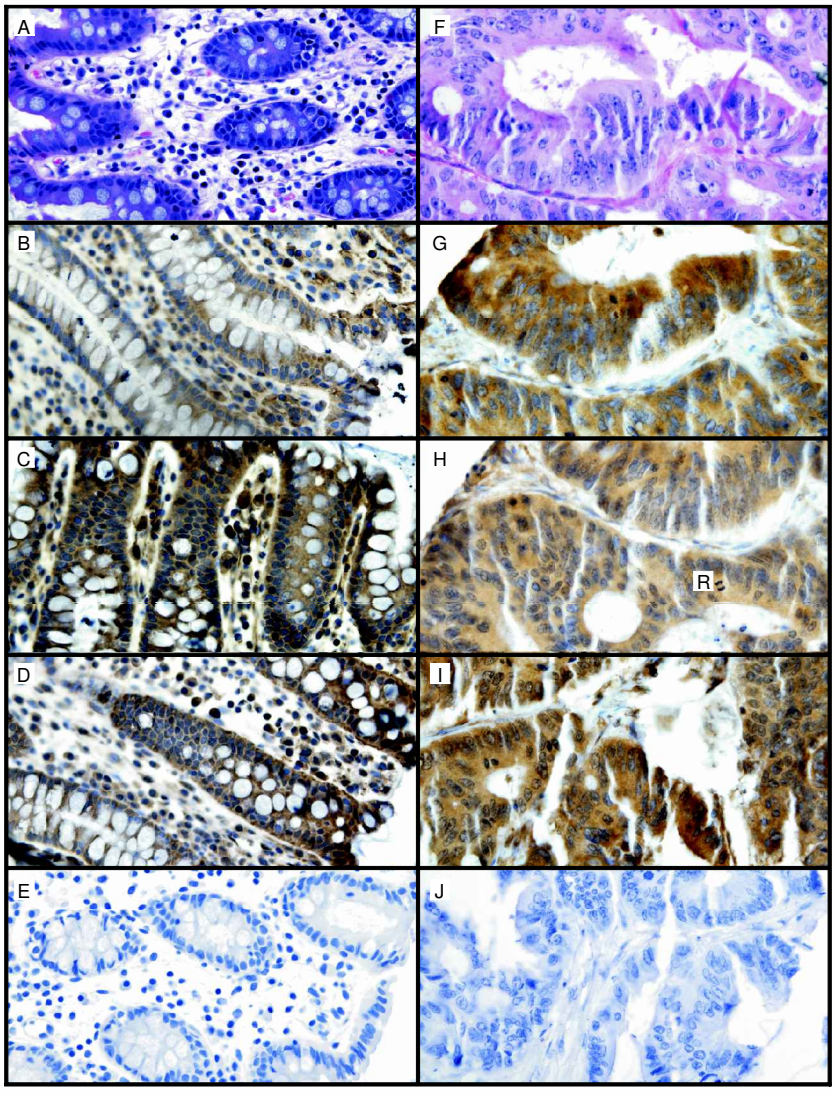

FIGURE 1 Smad expression and activation in normal human colonic mucosa and colorectal cancer. Consecutive 5-μm paraffin sections of normal colonic mucosa (A through E) and invasive colorectal cancer (F through J) were stained using hematoxylin-eosin (A through F), a polyclonal goat anti-Smad2 (S-20) antibody B and G), our polyclonal rabbit anti-pSmad2 antibody (C and H), or a mouse monoclonal anti-Smad4 antibody (B-8, Santa Cruz) (D and I), to detect expression of total Smad2, pSmad2, and Smad4, respectively. Control sections of each specimen were processed without primary antibodies (E and J). All sections were counterstained with hematoxylin. Representative areas of normal colonic mucosa (A–E) and invasive colon cancer (F–J) are shown (400× magnification). R, mitosis.

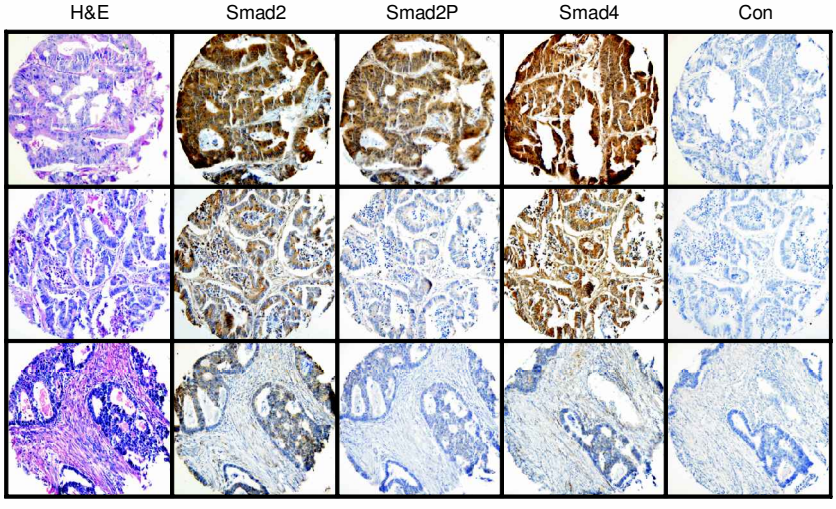

FIGURE 2 Smad expression and activation in human colorectal cancer. Consecutive 5-μm sections of two different tissue microarrays containing a total of 695 cases of primary invasive colorectal cancer were stained using a mouse monoclonal anti-Smad4 antibody (B-8, Santa Cruz), a polyclonal goat anti-Smad2 (S-20) antibody, or our polyclonal rabbit anti-pSmad2 antibody, to detect expression of Smad4, total Smad2, and pSmad2, respectively. Control sections were stained with the respective secondary antibody only (CON) or with hematoxylin-eosin (H&E). Representative 0.6-mm diameter sections of invasive colon carcinomas are shown (200× magnification). Top row: Most cases of invasive colorectal carcinomas expressed Smad4, Smad2, and pSmad2. Middle row: Representative case of a tumor that expressed Smad4 and Smad2 but failed to express pSmad2. Bottom row: Representative case of a tumor that expressed Smad2 but failed to express pSmad2 and Smad4.

TABLE 2 Smad Expression and Activation in Colorectal Cancer Tissue Microarrays: Three-Way Cross-Classification for Smad2, pSmad2, and Smad4[a]

		Smad4		
	pSmad2	Negative	Positive	*Total*
Smad2 Positive	Negative	3[b]	14	*17*
	Positive	4	554	*558*
Smad2 Negative		4[b]	7	*11*
Total		*11*	*575*	*586*

[a]Three-way cross-classification for Smad2, pSmad2, and Smad4. This analysis was performed on the subset of 586 cases for which immunostaining with all three antibodies could be reliably ascertained. Colorectal cancers displayed one of six phenotypes: Smad2, pSmad2, and Smad4 positive (554 cases; 94.5%); Smad2 negative (7 cases; 1%); pSmad2 negative (14 cases; 2%); Smad4 negative (4 cases; 0.7%); Smad2 and Smad4 negative (4 cases; 0.7%); and pSmad2 and Smad4 negative (3 cases; 0.5%).
[b]Cases with dual defect in transforming growth factor-β signaling.

prognostic and/or predictive significance.[18,49-54] We examined the possible relationships between loss of TGF-β signaling (as measured by pSmad2, Smad2, or Smad4 negativity) and overall survival in 624 unselected colorectal cancer cases for which this information was available (Fig. 3). As shown in Table 3 and Figure 3, the overall survival of patients whose cancers did not express any one of the TGF-β signaling intermediates (pSmad2, Smad2, or Smad4) or combinations thereof was significantly shorter than that of those whose tumors expressed Smad2, pSmad2, and Smad4 (median overall survival: 35 vs 58 months; $P = 0.0096$ by log-rank Mantel-Cox test; $P = 0.021$ by Peto and Peto's generalized Wilcoxon test). Moreover, the failure to express pSmad2 or either Smad2 or Smad4 individually was associated with a poor outcome, even though the observed differences did not quite reach statistical significance (Table 3). Thus, in the present series, any disruption in Smad signaling, whether it affected Smad2 or Smad4 expression or Smad2 activation, appears to define a small but particularly aggressive subset of colorectal cancers.

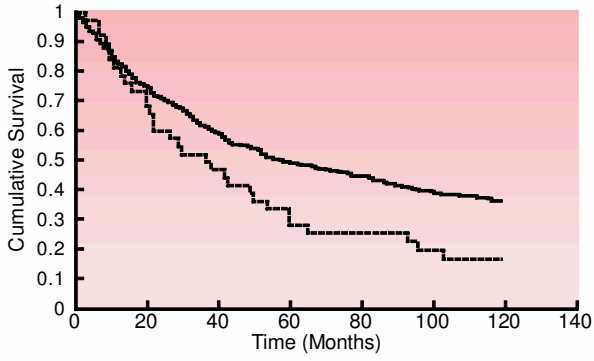

FIGURE 3 Relationship between loss of Smad signaling and clinical outcome. Survival curves were estimated according to the Kaplan-Meier method.[45] The curves represent overall survival of the patients in whose tumors we found evidence for loss of transforming growth factor-β (TGF-β)/Smad signaling (Smad negative) compared with the group in which we did not (Smad positive). The median overall survival of patients whose cancers failed to express Smad2, pSmad2, or Smad4 (35 months; standard error [SE], 12.3 months) was significantly shorter than of those whose tumors expressed Smad2, pSmad2, and Smad4 (58 months; SE, 4.9 months) (Chi-square = 6.715; $P = 0.0096$ by log-rank Mantel-Cox test; Chi-square = 5.325; $P = 0.021$ by Peto and Peto's generalized Wilcoxon test).

Associations Between Smad Expression and Pathological and Biologic Features of Colorectal Carcinoma

To identify possible associations between the patterns of expression of Smad2, pSmad2, and Smad4 and other clinical and pathological features of the colorectal carcinomas, Smad expression patterns were compared with tumor location, histologic grade, pathological stage, and presence or absence of lymph node metastases (Table 4). We found no association between Smad status and histological grade or tumor location. Interestingly, tumors with any type of loss of Smad signaling were more likely to be of more advanced pathological stage and to be associated with the presence of lymph node metastases (odds ratios: 2.15 and 2.35, respectively) (Table 4). Both advanced stage and presence of lymph node metastases are predictive of poor overall survival (Table 5). After adjustment for stage or presence of lymph node metastases, the relationship between the presence of a Smad signaling defect and overall survival was no longer significant (Table 5). Thus, our results indicate that TGF-β signaling defects in the primary tumor are strongly associated with the presence of metastatic disease at diagnosis and, hence, indirectly affect patient outcome.

DISCUSSION

The main purpose of this study was to assess the state of TGF-β signaling in colorectal cancer and its impact on patient outcome in a large cohort of archived cases for which this information was available. Although immunostainable TGF-β has been detected in and around colorectal cancers and the intensity of staining seems to correlate with advancing stages of tumor progression,[18,55-57] interpretation of these studies is complicated by difficulties associated with distinguishing the biologically inactive, latent form of TGF-β from its activated form. To circumvent these problems, we used phosphor-

TABLE 3 Effect of Smad Signaling Defects on Patient Overall Survival[a]

	Number	Survival[b] (Median ± SE)	Chi-Square	*P* Value
pSmad2				*0.0616*
Positive	566	55 ± 5.9		
Negative	21	47 ± 9.2	3.49	
Unscorable	25			
Smad2				*0.0471*
Positive	599	54 ± 8.1	3.94	
Negative	12	18 ± 6.1		
Unscorable	13			
Smad4			2.06	*0.1512*
Positive	582	57 ± 3.9		
Negative	13	41 ± 22.5		
Unscorable	29			
Combined[c]			6.715	*0.0096*
Positive	539	58 ± 4.9		
Negative	38	35 ± 12.3		
Unscorable	47			

[a]This analysis was performed on the subset of 624 cases for which overall survival information was available.
[b]Survival (in months) curves were estimated according to the Kaplan-Meier method,[45] and differences were assessed by means of the log-rank test (Mantel-Cox).
[c]Includes all cases that failed to express pSmad2, Smad2, or Smad4.

TABLE 4 Associations Between Smad Expression and Clinical and Pathological Features of Colorectal Carcinomas[a]

	Smad, *N*				
Variable	Positive	Negative	*P* Value[b]	Odds Ratio	95% CI
Histologic grade[b]	432	26	1.000	0.89	0.26–3.08
Low	377	23			
High	55	3			
Pathological stage[c]	506	40	0.031	2.15	1.11–4.18
Stages I + II	285	15			
Stages III + IV	221	20			
LNN metastases	498	38	0.015	2.35	1.20–4.58
Negative	314	16			
Positive	184	22			
Tumor location[d]	463	34	1.000	0.96	0.45–2.06
Proximal	132	10			
Distal	331	24			

Abbreviation: LNN, lymph node(s) negative.
[a]Contingency table analyses using the Fisher's exact test were used to determine the relationships between Smad status and known prognostic factors for colorectal carcinoma. Cases with incomplete information were excluded from the individual analyses.
[b]Histological grade: low, moderately and well-differentiated tumors; high, undifferentiated and poorly differentiated tumors.
[c]AJCC/UICC pathological stage.[79]
[d]Tumor location: proximal colon includes cecum, ascending colon, and transverse colon; distal colon includes splenic flexure, descending colon, sigmoid, and rectum.

ylation of the principal TβR-I substrate, Smad2, as a surrogate marker of activation of the TGF-β receptor system by TGF-β. In cultured cells, the pSmad2-specific antibody recognizes the phosphorylated form of Smad2 in a highly specific and sensitive manner, and that Smad2 phosphorylation occurs in response to TGF-β treatment in a dose- and time-dependent fashion.[41,42] Moreover, we have demonstrated good concordance between the expression of pSmad2 by immunohistochemistry and Western blotting of the same tissues (R. Ge,

TABLE 5 Associations Between Clinical, Pathological and Molecular Variables and Patient Overall Survival According to Proportional Hazards Regression Models[a]

Multiple Variables	Coefficient	SE	Chi-Square	*P* Value
Pathological stage[b] (adjusted for Smad signaling defect)	−0.494	0.095	27.025	< 0.0001
LNN metastases[c] (adjusted for Smad signaling defect)	0.413	0.098	17.877	< 0.0001
Smad signaling defect[d] (adjusted for stage)	−0.214	0.175	1.495	0.2214
Smad signaling defect[d] (adjusted for LNN metastases)	−0.219	0.180	1.481	0.2236

Abbreviations: SE, standard error.

[a]Proportional-hazards regression models were used for multivariable comparisons of time-to-event end points.[46] After adjustment for the presence of Smad signaling defects, pathological stage, and presence of lymph node metastases remained highly significant predictors of overall survival. However, after the analysis was adjusted for pathological stage and presence of lymph node metastases, Smad signaling defects no longer had a significant relation to overall survival.

[b]American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) pathological stages I and II versus III and IV.[79]

[c]Positive versus negative

[d]Positive versus negative.

M. Reiss, 2002, unpublished observation). Thus, the presence of pSmad2 in cells can be used as an indicator of receptor signaling by biologically active TGF-β present within the cellular microenvironment.

Our results indicate that the microenvironment of the normal colonic mucosa apparently contains biologically active TGF-β, leading to activation of Smad2 in epithelial, stromal, and capillary structures. This finding was somewhat unexpected, because TGF-β is generally believed to be deposited in the extracellular milieu in a latent form and not to become activated except in response to tissue injury.[58] In addition, we observed a gradient of pSmad2 along the crypt axis, increasing from the bottom of the crypts to the luminal surface. This apparent gradient of Smad2 activation is consistent with the pattern of immunostaining for TGF-β1 described by Avery et al.[59]

Somewhat to our surprise, more than 90% of colorectal carcinomas expressed pSmad2. This indicates that bioactive TGF-β is present in the microenvironment and is inducing receptor signaling in these cases. In addition, and perhaps more importantly, this observation provides direct evidence that the malignant tumor cells are, in fact, capable of proliferating in the presence of biologically active TGF-β in vivo. In this respect, the phenotype of colorectal carcinomas in vivo parallels that of most colon cancer cell lines in vitro.[60] Moreover, this finding supports the idea that TGF-β provides a selective pressure that favors the outgrowth of cell clones that are resistant to TGF-β–mediated cell cycle arrest.

Using tumor cell lines, we have shown that failure of TGF-β to induce phosphorylation of Smad2 accurately reflects complete loss of expression or presence of an inactivating mutation of one of the TGF-β receptors.[41,42] Thus, as long as active TGF-β is present, loss of pSmad2 immunostaining can be used as a surrogate marker for a TGF-β receptor defect in vivo.[41] In the present series, approximately 6% of colorectal cancers failed to express pSmad2. In two thirds of these cases, this was clearly due to a lack of phosphorylation and not due to a loss of Smad2 expression. In addition, it was not due to absence of ligand, because surrounding normal cells continued to express pSmad2. Thus, selective loss of Smad2 activation is most likely due to a defect in receptor signaling.[41,42] The frequency of pSmad2-negativity in the present study is very similar to the frequency of pSmad2-negativity we encountered in human breast cancers[41] but significantly lower than in endometrial cancers.[43] Thus, the frequency of TGF-β receptor signaling loss varies considerably between different cancer types.

In colorectal cancers associated with microsatellite instability, mutations found within the TβR-II coding sequence result in mRNA instability and loss of receptor expression.[27,61] As microsatellite instability is found in approximately 15% of unselected colorectal cancers,[62] and about one half to two thirds of these carry *TβR-II* mutations,[33,54] it is possible that the pSmad2-negative cases in our series are representative of the RER+ phenotype. Because only complete absence of TβR-II would result in loss of TGF-β responsiveness,[63] our findings suggest that this degree of reduction in TGF-β receptor expression occurs in no more than 4% of unselected colorectal carcinomas. This conclusion is entirely consistent with reports that complete loss of TβR messenger RNA expression occurs in a small fraction of colon carcinomas[64] and of colon carcinoma cell lines.[61,65] Besides loss of receptor expression, loss-of-function receptor mutations would also result in pSmad2 negativity.[42] *TβR-II* mutations are frequently found in RER+ colo-

rectal cancers and in a small fraction of microsatellite stable colon cancers.[40] Thus, it is likely that the selective absence of Smad2 activation we observed in approximately 4% of colorectal cancer specimens reflects the presence of inactivating mutations and/or loss of expression of the *TβR-II* gene.

Only 2% of our cases of colorectal cancer failed to express Smad2 protein. This is consistent with a reported frequency of *Smad2* genomic alterations in colorectal cancer of between 1% and 5%.[21,23,66-68] However, these genomic alterations have included mainly missense mutations of the *Smad2* gene and only a small number of allelic deletions, which may not all result in loss of protein expression. Thus, additional mechanisms of gene inactivation may be responsible for loss of Smad2 protein.

Wilentz et al[47,48] recently demonstrated that loss of Smad4 immunostaining using the B-8 antibody accurately reflects loss or intragenic mutation of both alleles of the Smad4 gene. In the present study, only 2.3% of the colorectal cancers failed to express Smad4 protein. This is significantly lower than the estimated 15%–30% of Smad4 protein negativity previously reported in two small studies of a total of 94 cases.[26,69] That the frequency of Smad4 protein negativity is lower than the estimated frequency of Smad4 genomic alterations in colorectal cancers[20,22,24,25,67,68,70,71] is likely due to the fact that allelic loss not always denotes inactivation of both alleles, and that not all Smad4 mutations result in loss of protein expression.

Interestingly, we encountered a small number of cases of colorectal cancer with a dual defect (loss of both pSmad2 and Smad4, or both Smad2 and Smad4). It is possible that inactivation of TGF-β receptors or Smad2 on the one hand and of Smad4 on the other confer partially nonoverlapping selective advantages during tumor development. This idea is supported by numerous recent reports of Smad4-negative human tumor cell lines in which one of the TGF-β receptor subtypes was also inactivated.[40,72]

Ours is the largest series of colorectal cancers in which Smad signaling has been examined to date and the one with the longest follow-up. One of the most important findings of the present study is the fact that the overall survival of patients whose cancers displayed any of the defects in Smad activation or expression was significantly shorter than that of those whose cancers expressed Smad2, pSmad2, and Smad4. This is consistent with previous reports that allelic loss of chromosome 18q21 has a negative impact on prognosis.[49,50,52,53,73-76] Although this region encompasses both the Smad4 and Smad2 loci, the frequency of *Smad2* or *Smad4* gene losses is much lower than that of allelic loss at 18q21, suggesting that additional putative tumor suppressor genes in this area (e.g., *DCC*) may be important. Ours is the first study to demonstrate that inactivation of Smad2 and Smad4 per se (as determined by lack of protein expression) is associated with poor prognosis. Although Watanabe et al[54] have suggested that 18q21 loss is predictive of poor outcome in response to adjuvant chemotherapy in patients with stage III and high-risk stage II disease, most of our cases predate the widespread use of adjuvant therapy and were unselected in terms of stage. Thus, our observed effect of Smad loss on overall survival is likely independent of the effect of chemotherapy.

The effect on prognosis was most clearly seen when all cases with Smad signaling defects were pooled. However, each individual lesion (loss of pSmad2, Smad2, or Smad4 expression) was associated with a negative outcome, although the numbers of cases were too small to achieve statistical significance. Although it is biologically plausible that any defect in TGF-β signaling would have a similar effect on the tumors, our results might seem to be at odds with studies that showed that microsatellite unstable colorectal cancers have an intrinsically lower tendency to metastasize and better outcome. Although 50%–60% of these RER+ colon cancers carry inactivating *TβR-II* mutations, our results suggest that it may be other genetic changes associated with the microsatellite instability syndrome (e.g., *BAX* inactivation) that confer a good prognosis on RER+ cases. Moreover, microsatellite unstable cases are usually found in the proximal colon, whereas in our series, there was no indication that any of the Smad defects had a particular predilection for the proximal versus distal colon. Although a recent study[54] seems to indicate that the presence of *TβR-II* microsatellite mutations is predictive of better outcome after adjuvant chemotherapy, all patients received chemotherapy, so it is difficult to distinguish intrinsic biologically favorable effects from chemotherapy-specific effects.

In our study, the presence of Smad defects was clearly associated with higher pathological stage and presence of lymph node metastases. After the analysis was adjusted for stage or presence of lymph node metastases, the relationship between the presence of a Smad signaling defect and patient overall survival was no longer statistically significant (Table 5). Thus, although TGF-β signaling defects in the primary tumor are clearly associated with poor patient outcome, this appears to be a function of their association with metastatic disease at diagnosis. In this respect, the results of the current study are consistent with those of several smaller previous studies that have reported an association between Smad4 genomic alterations in colorectal cancer and metastatic spread.[67,68,71,77]

Conversely, in two other recent analyses of human

pancreatic and breast cancers, the negative impact of Smad signaling defects on patient outcome was independent of stage and metastatic spread.[41,78] Future studies will need to address in more detail how alterations in TGF-β signaling affect the biology of human cancer and its response to treatment. Meanwhile, assessing the state of TGF-β signaling in colorectal cancer biopsies may be useful in treatment planning, because of its ability to predict both the extent of disease before surgery and, possibly, the need for adjuvant systemic therapy.

ACKNOWLEDGMENTS

We wish to thank Ms. Lori A. Charette for her expert assistance in the preparation and processing of the tissue microarrays. This work was supported, in part, by grants from the National Cancer Institute, the Patrick and Catherine Weldon-Donaghue Medical Research Foundation, and the Anna Hull Fund.

REFERENCES

1. Roberts AB, Anzano MA, Wakefield LM et al. Type β transforming growth factor: a bifunctional regulator of cellular growth. Proc Natl Acad Sci USA 1985;82:119–123.
2. Massagué J. TGF β signal transduction. Annu Rev Biochem 1998; 67:753–791.
3. Kurokowa M, Lynch K, Podolsky DK. Effects of growth factors on an intestinal epithelial cell line: transforming growth factor beta inhibits proliferation and stimulates differentiation. Biochem Biophys Res Commun 1987;142:775–782.
4. Winesett MP, Ramsey GW, Barnard JA. Type II TGF(beta) receptor expression in intestinal cell lines and in the intestinal tract. Carcinogenesis 1996;17:989–995.
5. Ko TC, Beauchamp RD, Townsend CM Jr et al. Transforming growth factor-beta inhibits rat intestinal cell growth by regulating cell cycle specific gene expression. Am J Surg 1994;167:14–19; discussion 19–20.
6. Ko TC, Sheng HM, Reisman D et al. Transforming growth factor-beta 1 inhibits cyclin D1 expression in intestinal epithelial cells. Oncogene 1995;10:177–184.
7. Ko TC, Yu W, Sakai T et al. TGF-beta1 effects on proliferation of rat intestinal epithelial cells are due to inhibition of cyclin D1 expression. Oncogene 1998;16:3445–3454.
8. Ciacci C, Lind SE, Podolsky DK. Transforming growth factor beta regulation of migration in wounded rat intestinal epithelial monolayers. Gastroenterology 1993;105:93–101.
9. Dignass AU, Podolsky DK. Cytokine modulation of intestinal epithelial cell restitution: central role of transforming growth factor beta. Gastroenterology 1993;105:1323–1332.
10. McKaig BC, Makh SS, Hawkey CJ et al. Normal human colonic subepithelial myofibroblasts enhance epithelial migration (restitution) via TGF-beta3. Am J Physiol 1999;276:G1087–1093.
11. Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. Crit Rev Oncog 1999;10:303–360.
12. Zhu Y, Richardson JA, Parada LF et al. *Smad3* mutant mice develop metastatic colorectal cancer. Cell 1998;94:703–714.
13. Takaku K, Oshima M, Miyoshi H et al. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. Cell 1998;92:645–656.
14. Takaku K, Miyoshi H, Matsunaga A et al. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. Cancer Res 1999;59: 6113–6117.
15. Xu X, Brodie SG, Yang X et al. Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. Oncogene 2000;19:1868–1874.
16. Howe JR, Roth S, Ringold JC et al. Mutations in the SMAD4/DPC4 gene in juvenile polyposis [see comments]. Science 1998;280: 1086–1088.
17. Lu SL, Kawabata M, Imamura T et al. HNPCC associated with germline mutation in the TGF-beta type II receptor gene. Nat Genet 1998;19:17–18.
18. Friedman E, Gold LI, Klimstra D et al. High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. Cancer Epidemiol Biomarkers Prev 1995;4:549–554.
19. Laurent-Puig P, Blons H, Cugnenc PH. Sequence of molecular genetic events in colorectal tumorigenesis. Eur J Cancer Prev 1999; 8[suppl 1]:S39–47.
20. Riggins GJ, Kinzler KW, Vogelstein B et al. Frequency of Smad gene mutations in human cancers. Cancer Res 1997;57:2578–2580.
21. Takagi Y, Koumura H, Futamura M et al. Somatic alterations of the SMAD-2 gene in human colorectal cancers. Br J Cancer 1998; 78:1152–1155.
22. Takagi Y, Kohmura H, Futamura M et al. Somatic alterations of the DPC4 gene in human colorectal cancers in vivo. Gastroenterology 1996;111:1369–1372.
23. Eppert K, Scherer SW, Ozcelik H et al. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell 1996;86: 543–552.
24. Thiagalingam S, Lengauer C, Leach FS et al. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nat Genet 1996;13:343–346.
25. MacGrogan D, Pegram M, Slamon D et al. Comparative mutational analysis of DPC4 (Smad4) in prostatic and colorectal carcinomas. Oncogene 1997;15:1111–1114.
26. Korchynskyi O, Landstrom M, Stoika R et al. Expression of Smad proteins in human colorectal cancer. Int J Cancer 1999;82: 197–202.
27. Markowitz S, Wang J, Myeroff L et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability [see comments]. Science 1995;268:1336–1338.
28. Myeroff LL, Parsons R, Kim S-J et al. A transforming growth factor β type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res 1995;55:5545–5547.
29. Parsons R, Myeroff LL, Liu B et al. Microsatellite instability and mutations of the transforming growth factor β type II receptor gene in colorectal cancer. Cancer Res 1995;55:5548–5550.
30. Wang J, Sun L, Myeroff L et al. Demonstration that mutation of the type II transforming growth factor beta receptor inactivates its tumor suppressor activity in replication error-positive colon carcinoma cells. J Biol Chem 1995;270:22044–22049.
31. Lu SL, Akiyama Y, Nagasaki H et al. Mutations of the transforming growth factor-beta type II receptor gene and genomic instability in hereditary nonpolyposis colorectal cancer. Biochem Biophys Res Commun 1995;216:452–457.
32. Abe T, Ouyang H, Migita T et al. The somatic mutation frequency of the transforming growth factor beta receptor type II gene varies widely among different cancers with microsatellite instability. Eur J Surg Oncol 1996;22:474–477.
33. Akiyama Y, Iwanaga R, Ishikawa T et al. Mutations of the transforming growth factor-beta type II receptor gene are strongly related to sporadic proximal colon carcinomas with microsatellite instability. Cancer 1996;78:2478–2484.
34. Ohue M, Tomita N, Monden T et al. Mutations of the transforming

growth factor beta type II receptor gene and microsatellite instability in gastric cancer. Int J Cancer 1996;68:203–206.
35. Lu SL, Zhang WC, Akiyama Y et al. Genomic structure of the transforming growth factor beta type II receptor gene and its mutations in hereditary nonpolyposis colorectal cancers. Cancer Res 1996;56:4595–4598.
36. Konishi M, Kikuchi-Yanoshita R, Tanaka K et al. Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer [see comments]. Gastroenterology 1996;111:307–317.
37. Renault B, Calistri D, Buonsanti G et al. Microsatellite instability and mutations of p53 and TGF-beta RII genes in gastric cancer. Human Gen 1996;98:601–607.
38. Togo G, Toda N, Kanai F et al. A transforming growth factor beta type II receptor gene mutation common in sporadic cecum cancer with microsatellite instability. Cancer Res 1996;56:5620–5603.
39. Samowitz WS, Slattery ML. Transforming growth factor-beta receptor type 2 mutations and microsatellite instability in sporadic colorectal adenomas and carcinomas. Am J Pathol 1997;151: 33–35.
40. Grady WM, Myeroff LL, Swinler SE et al. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. Cancer Res 1999;59:320–324.
41. Xie W, Mertens JC, Reiss DJ et al. Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. Cancer Res 2002;62:497–505.
42. Yan W, Vellucci VF, Reiss M. Smad protein expression and activation in transforming growth factor-beta refractory human squamous cell carcinoma cells. Oncol Res 2000;12:157–167.
43. Parekh TV, Gama P, Wen X et al. Transforming growth factor beta signaling is disabled early in human endometrial carcinogenesis concomitant with loss of growth inhibition. Cancer Res 2002;62: 2778–2790.
44. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000;80:1943–1949.
45. Kaplan EL, Meier P. Nonparametric estimation from incomplete observation. J Am Statist Assoc 1958;53:457–481.
46. Cox DR. Regression models and life-tables. J Royal Stat Soc 1972; 34:187–202.
47. Wilentz RE, Iacobuzio-Donahue CA, Argani P et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res 2000;60:2002–2006.
48. Wilentz RE, Su GH, Dai JL et al. Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas: a new marker of DPC4 inactivation. Am J Pathol 2000;156:37–43.
49. Kern SE, Fearon ER, Tersmette KW et al. Clinical and pathological associations with allelic loss in colorectal carcinoma [corrected]. JAMA 1989;261:3099–3103.
50. Jen J, Kim H, Piantadosi S et al. Allelic loss of chromosome 18q and prognosis in colorectal cancer. N Engl J Med 1994;331: 213–221.
51. Bubb VJ, Curtis LJ, Cunningham C et al. Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. Oncogene 1996;12:2641–2649.
52. Lanza G, Matteuzzi M, Gafa R et al. Chromosome 18q allelic loss and prognosis in stage II and III colon cancer. Int J Cancer 1998; 79:390–395.
53. Ogunbiyi OA, Goodfellow PJ, Herfarth K et al. Confirmation that chromosome 18q allelic loss in colon cancer is a prognostic indicator. J Clin Oncol 1998;16:427–433.
54. Watanabe T, Wu TT, Catalano PJ et al. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. N Engl J Med 2001;344:1196–1206.
55. Cardillo MR, Yap EP. TGF-beta 1 in colonic neoplasia: a genetic molecular and immunohistochemical study. Arch Anat Cytol Pathol 1996;44:241–249.
56. Tsushima H, Kawata S, Tamura S et al. High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. Gastroenterology 1996;110:375–382.
57. Picon A, Gold LI, Wang J et al. A subset of metastatic human colon cancers expresses elevated levels of transforming growth factor beta 1. Cancer Epidemiol Biomarkers Prev 1998;7:497–504.
58. Koli K, Saharinen J, Hyytiainen M et al. Latency, activation, and binding proteins of TGF-beta. Microsc Res Tech 2001;52:354–362.
59. Avery A, Paraskeva C, Hall P et al. TGF-beta expression in the human colon: differential immunostaining along crypt epithelium. Br J Cancer 1993;68:137–139.
60. Fynan TM, Reiss M. Resistance to inhibition of cell growth by transforming growth factor-β and its role in oncogenesis. Crit Rev Oncogen 1993;4:493–540.
61. Jiang W, Tillekeratne MP, Brattain MG et al. Decreased stability of transforming growth factor beta type II receptor mRNA in RER+ human colon carcinoma cells. Biochemistry 1997;36: 14786–14793.
62. Boland CR, Thibodeau SN, Hamilton SR et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248–5257.
63. Ko Y, Banerji SS, Liu Y et al. Expression of transforming growth factor-beta receptor type II and tumorigenicity in human breast adenocarcinoma MCF-7 cells. J Cell Physiol 1998;176:424–434.
64. Matsushita M, Matsuzaki K, Date M et al. Down-regulation of TGF-beta receptors in human colorectal cancer: implications for cancer development. Br J Cancer 1999;80:194–205.
65. Wang J, Han W, Zborowska E et al. Reduced expression of transforming growth factor β type I receptor contributes to the malignancy of human colon carcinoma cells. J Biol Chem 1996;271: 17366–17371.
66. Takenoshita S, Tani M, Mogi A et al. Mutation analysis of the Smad2 gene in human colon cancers using genomic DNA and intron primers. Carcinogenesis 1998;19:803–807.
67. Miyaki M, Iijima T, Konishi M et al. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. Oncogene 1999;18:3098–3103.
68. Ohtaki N, Yamaguchi A, Goi T et al. Somatic alterations of the DPC4 and Madr2 genes in colorectal cancers and relationship to metastasis. Int J Oncol 2001;18:265–270.
69. Salovaara R, Roth S, Loukola A et al. Frequent loss of SMAD4/DPC4 protein in colorectal cancers. Gut 2002;51:56–59.
70. Schutte M, Hruban RH, Hedrick L et al. DPC4 gene in various tumor types. Cancer Res 1996;56:2527–2530.
71. Tarafa G, Villanueva A, Farre L et al. DCC and SMAD4 alterations in human colorectal and pancreatic tumor dissemination. Oncogene 2000;19:546–555.
72. Dai JL, Turnacioglu KK, Schutte M et al. Dpc4 transcriptional activation and dysfunction in cancer cells. Cancer Res 1998;58: 4592–4597.
73. Carethers JM, Hawn MT, Greenson JK et al. Prognostic significance of allelic lost at chromosome 18q21 for stage II colorectal cancer. Gastroenterology 1998;114:1188–1195.
74. Martinez-Lopez E, Abad A, Font A et al. Allelic loss on chromosome 18q as a prognostic marker in stage II colorectal cancer. Gastroenterology 1998;114:1180–1187.
75. Jernvall P, Makinen MJ, Karttunen TJ et al. Loss of heterozygosity at 18q21 is indicative of recurrence and therefore poor prognosis in a subset of colorectal cancers. Br J Cancer 1999;79:903–908.
76. Chan TL, Curtis LC, Leung SY et al. Early-onset colorectal cancer with stable microsatellite DNA and near-diploid chromosomes. Oncogene 2001;20:4871–4876.

77. Maitra A, Molberg K, Albores-Saavedra J et al. Loss of Dpc4 expression in colonic adenocarcinomas correlates with the presence of metastatic disease. Am J Pathol 2000;157:1105–1111.
78. Tascilar M, Skinner HG, Rosty C et al. The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. Clin Cancer Res 2001;7:4115–4121.
79. Greene FL, Page DL, Fleming ID et al. AJCC Cancer Staging Handbook, ed. 6. New York: Springer, 2002.