Translational Cancer Mechanisms and Therapy

Clinical Cancer Research

SMAD4 Loss in Colorectal Cancer Patients Correlates with Recurrence, Loss of Immune Infiltrate, and Chemoresistance

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Abstract

Purpose: SMAD4 has shown promise in identifying patients with colorectal cancer at high risk of recurrence or death.

Experimental Design: A discovery cohort and independent validation cohort were classified by SMAD4 status. SMAD4 status and immune infiltrate measurements were tested for association with recurrence-free survival (RFS). Patient-derived xenografts from SMAD4-deficient and SMAD4-retained tumors were used to examine chemoresistance.

Results: The discovery cohort consisted of 364 patients with stage I–IV colorectal cancer. Median age at diagnosis was 53 years. The cohort consisted of 61% left-sided tumors and 62% stage II/III patients. Median follow-up was 5.4 years (interquartile range, 2.3–8.2). SMAD4 loss, noted in 13% of tumors, was associated with higher tumor and nodal stage, adjuvant therapy use, fewer tumor-infiltrating lymphocytes (TIL), and lower peritumoral lymphocyte aggregate (PLA) scores (all $P < 0.04$). SMAD4 loss was associated with worse RFS ($P = 0.02$). When stratified by SMAD4 and immune infiltrate status, patients with SMAD4 loss and low TIL or PLA had worse RFS ($P = 0.002$ and $P = 0.006$, respectively). Among patients receiving 5-fluorouracil (5-FU)-based systemic chemotherapy, those with SMAD4 loss had a median RFS of 3.8 years compared with 13 years for patients with SMAD4 retained. In xenografted mice, the SMAD4-lost tumors displayed resistance to 5-FU. An independent cohort replicated our findings, in particular, the association of SMAD4 loss with decreased immune infiltrate, as well as worse disease-specific survival.

Conclusions: Our data show SMAD4 loss correlates with worse clinical outcome, resistance to chemotherapy, and decreased immune infiltrate, supporting its use as a prognostic marker in patients with colorectal cancer.

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Translational Relevance

SMAD4 loss, when noted in tumors of patients with colorectal cancer, is a potentially useful marker identifying patients at high risk of recurrence and death. Our study, utilizing both discovery and independent validation cohorts, along with complementary *in vivo* data, strengthens this association by showing that SMAD4 loss correlates with worse survival and with resistance to chemotherapy. Moreover, our study describes the association of SMAD4 loss with decreased tumor immune infiltrate, perhaps implicating a role for SMAD4 in the immune environment in colorectal cancer pathogenesis.

Introduction

Despite advances in screening and treatment, colorectal cancer persists as the second leading cause of cancer-related deaths in the United States (1). As new treatments become available, the identification of both prognostic and predictive markers is of the utmost importance. One approach for stratifying patients with colorectal cancer into specific treatment approaches is the use of tissue biomarkers (2). A promising candidate is SMAD4, a tumor suppressor that is the central node in TGFβ signaling.

SMAD4 is involved in the regulation of cell proliferation, differentiation, migration, and apoptosis (3). Mediating the cross-talk between epithelial and stromal inflammatory cells, SMAD4 loss can lead to the development of epithelial tumors in the gastrointestinal (GI) tract (4). Germline mutations of SMAD4 are found in over 50% of patients with familial juvenile polyposis, predisposing patients to hamartomatous polyps and GI cancer (4). Moreover, SMAD4 is mutated or altered in almost 13% of sporadic colorectal cancer cases (5).

SMAD4 loss has been associated with worse outcomes and hints to a predisposition to chemoresistance (6, 7), but no study has examined the relationship between SMAD4 and immune infiltration into colorectal tumors. Therefore, we explored the impact of SMAD4 loss on chemoresistance, tumor immune infiltration, and recurrence-free survival (RFS) in a large cohort of patients with colorectal cancer treated at a tertiary cancer care center with mature follow-up. We also sought to validate our findings in an independent cohort of over 200 colorectal tumors and to corroborate our clinical findings in a patient-derived xenograft model.

Materials and Methods

Discovery cohort

Inclusion criteria. Patients with surgically resected, primary colorectal cancer treated at Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY) were eligible for inclusion. Two arrays that had been created to assess mismatch repair status and potential biomarkers from a program project grant were combined. Patients were excluded if they had familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer. Only patients with accessible tissue blocks and clinical follow-up were included, resulting in a study cohort of 364 patients. Clinical and pathologic features, including treatment data, were extracted from the electronic medical records of patients. The study was approved by the institutional review board at MSKCC (New York, NY). A waiver was obtained from the Institutional Review Board at MSKCC (New York, NY) to identify patient records for review, identification, and retrieval of relevant clinical and pathologic information for this study.

Study power. To detect a hazard ratio (HR) of 1.25 with a delta of 1.4, assuming an overall cohort-wide probability of recurrence of 0.25 and proportion of SMAD4 loss of 0.13, a cohort of 219 patients would be sufficient to give a power of 0.80 with an alpha of 0.05.

Histology. Formalin-fixed, paraffin-embedded (FFPE) material was collected from each resected tumor. Each case was graded by a GI pathologist (L.H. Lee or J. Shia). Whole sections stained with hematoxylin and eosin (H&E) were assessed under high-power (40×0.2375 mm^2) magnification for peritumoral lymphocyte aggregates (PLA) and tumor-infiltrating lymphocytes (TIL). PLA were scored as none, few, or many per high-power field; TIL score was calculated as 0 (none), 1 (<15), 2 (15–215), or 3 (>215 per high-power field). Immune infiltrate was reviewed and scored by two GI pathologists using previously described methods (8, 9).

Immunohistochemistry. Colorectal cancer tissue microarrays (TMA) were constructed from surgically resected specimens of the 364 patients. These specimens were from the primary resections, prior to any evidence of recurrence of disease. The TMAs had a core size of 0.6 mm with three cores and were subjected to immunohistochemistry (IHC) for SMAD4 and mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, and PMS2). A rabbit mAb to SMAD4 (clone EP618Y from Abcam) was used in a 1:200 dilution. Mouse antibodies to MLH1 (clone M1 from Ventana), MSH2 (clone G219.1129 from Cell Marque), MSH6 (clone 44 from Ventana), and PMS2 (clone A16.4 from BD Biosciences) were used. Machine-based IHC was performed using Ventana XT Autostainer (Ventana Medical Systems, Inc.). SMAD4 nuclear staining intensity was reviewed and scored independently by two GI pathologists on a scale of 0 (absent) to 3 (strongly present). Disagreements in scoring were reconciled to reach a consensus. For the purposes of analysis, SMAD4 loss was defined as a score of 0; SMAD4 retained was defined as a score of 1–3. MMR proteins were scored as absent or present. MMR deficient was defined as absence of one or more of the MMR proteins. The MMR stains were done on TMAs; however, cases with negative or equivocal expression were repeated on whole slides for more accurate evaluation.

Statistical analysis. Student *t* test was used to assess the association of SMAD4 status and age. χ^2 tests (or Fisher exact test, depending on data distribution) were used to assess the association of SMAD4 status with clinical/pathologic features (tumor location, MMR results, TIL and PLA scores, and 5-FU–based systemic chemotherapy).

RFS was assessed using chart review and was defined as the time from initial resection to the documentation of recurrence or death, whichever came first. Survival curves by SMAD4 status for RFS were constructed using Kaplan–Meier estimates and compared using log-rank tests. To adjust for confounding variables, a forward stepwise Cox proportional hazards model was created. Student *t* test was used to assess tumor weight with 5-FU versus vehicle in the patient-derived tumoroids. Multivariate ANOVA

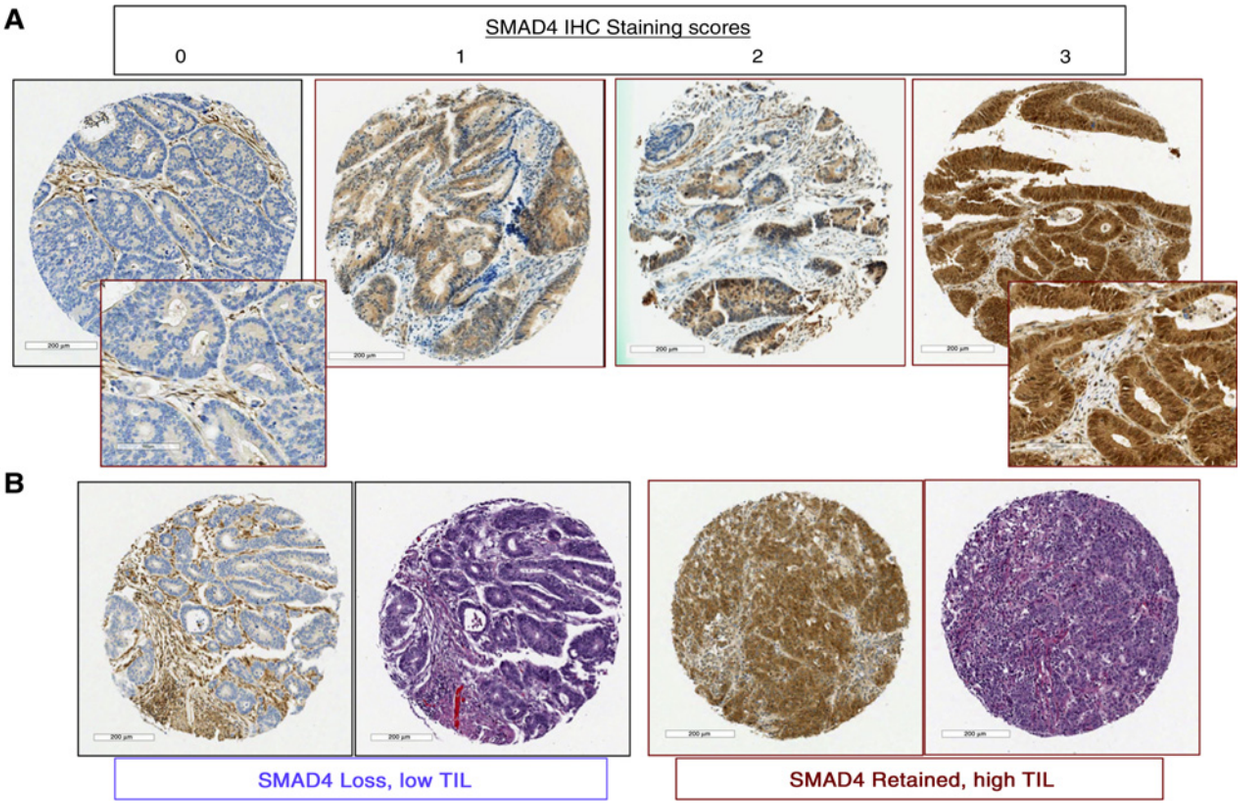

Figure 1.

Scoring of IHC results for SMAD4 and TILs in the discovery cohort. **A,** Representative IHC staining for each SMAD4 score. SMAD4 loss was defined as a score of 0 (left image), and SMAD4 retention was defined as a score of 1–3 (right three images). The magnification bars are 200 microns in the main images and 100 microns in the inset images. **B,** Representative H&E images for low and high TIL scores. Each H&E-stained image is shown next to the SMAD4 IHC staining from the same tumor core. Scale bars, 200 μm.

was used to compare tumoroid response to treatment. All statistical analyses were performed using SAS software (version 9.4; SAS Institute), and all *P* values were two-sided.

Independent validation cohort

In collaboration with the Harvard T.H. Chan School of Public Health, Dana-Farber Cancer Institute, and Brigham and Women's Hospital (Boston, MA), we independently identified 224 cases with available data on SMAD4 expression status in colorectal cancer tissue samples from two prospective cohort studies: The Nurses' Health Study (NHS, 121,701 women ages 30–55 years followed since 1976), and the Health Professionals Follow-up Study (HPFS, 51,529 men ages 40–75 years followed since 1986). FFPE tumor tissue blocks from hospitals throughout the United States where patients with colorectal cancer had undergone surgical resection were obtained and TMAs were created. Tumor and normal DNA was extracted, and MMR status was determined as described previously (10).

As reported previously (11), we constructed TMAs of colorectal cancers with sufficient tissue materials, including up to four tumor cores in one TMA block (each ~600 μm in diameter) from each case. IHC analysis for SMAD4 using an anti-SMAD4 antibody (clone B-8, dilution 1:100; Santa Cruz Biotechnology) was performed, and SMAD4 expression status was classified as intact, if there was nuclear expression anywhere within the tumor; or lost, if there was complete lack of expression in the tumor. In the validation cohort, the study pathologist (S. Ogino) evaluated histopathologic features including PLA and TIL of all included cases, and a second pathologist (J.N. Glickman) performed an independent review in selected cases with good agreement statistics, as described previously (12).

Characteristics between subgroups were compared using the Spearman correlation test for immune variables, the χ^2 test for other categorical variables, and an unpaired *t* test for continuous variables. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and compared using the log-rank test. All statistical analyses were performed using SAS software (version 9.4; SAS Institute), and all *P* values were two-sided.

Analyses using patient-derived tumoroids

We derived tumoroids from four freshly resected colorectal tumors to determine chemoresistance in tumors implanted in an ectopic model of tumorigenicity and in *ex vivo* chemosensitivity assays. The patient-derived tumoroids were known to have either a SMAD4 mutation or to be SMAD4 wild-type by Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT; ref. 13), and confirmed by Western blot analysis (see below). Specifically, the fresh tumors were dissociated into cells, then suspended in Matrigel (Growth Factor Reduced; BD Biosciences), and dispensed into a 24-well

suspension plate (50 μL/mL) to grow into tumoroids. After Matrigel polymerization, the tumoroids were overlaid with 0.5 mL of culture medium and maintained at 37°C with 5% CO_2. Media were changed every 2 or 3 days. The basal culture medium used for derivation of the patient-derived tumor cells was advanced DMEM/F12 medium supplemented with antibiotic–antimycotic solution (Gibco), 1 × B27, 1 × N2, 2 mmol/L GlutaMax (Invitrogen), 10 nmol/L gastrin I, 10 mmol/L HEPES, 1 mmol/L N-acetylcysteine, and 10 mmol/L nicotinamide (Sigma-Aldrich). The following niche factors were used: 25% Wnt-3A conditioned medium, 10% R-spondin conditioned medium, 50 ng/mL mouse recombinant Noggin (PeproTech), 50 ng/mL mouse recombinant EGF (Invitrogen), 500 nmol/L A83-01 (Tocris), and 10 μmol/L SB 202190 (Sigma-Aldrich). Colorectal cancer tumoroids were treated with designated concentrations of 5-FU or FOLFOX (5-FU + leucovorin + oxaliplatin, 25:5:1 molar ratio; 0, 0.5, 1, 5, 10, 50 μmol/L). The Matrigel was then dissolved using cell recovery solution, and a CellTiter Glo viability assay was performed to assess cell viability, and percent live cells were calculated and reported from multiple biologic replicates.

Western blots. Patient-derived samples were processed and lysed as described previously (14). Colorectal cancer tumoroid samples were processed according to published methods (15). Equal amounts of protein were loaded in each lane of a SDS–4%–12% polyacrylamide gel. Western blot analysis was performed by the standard method using the following primary antibodies: anti-SMAD4 (ab40759; Abcam; 1:1,000) and anti-β-actin (ab49900; Abcam; 1:10,000). Western blot images were analyzed, and bands quantified with ImageJ software (version 1.50b; NIH, Bethesda, MD; https://imagej.nih.gov/ij/).

Cell culture, cell viability, and immunoblot analysis. The human colon cancer cells SW480 and SW480^{SMAD4} (SW480 with SMAD4 restored) were generated and maintained as described previously (16). These cells were cultured in RPMI1640 containing 10% FBS and 1% antibiotic and antimycotic (Thermo Fisher Scientific). Cells were treated with 5-FU alone or FOLFOX at different concentrations (1×, 2×, and 4×; Supplementary Table S1) for 72 hours. Immunoblot analyses were performed using standard protocols as described previously (16). To assess cell viability, MTT assays were performed as described previously (16).

Animal studies. Patient-derived tumoroids (from one SMAD4-retained tumor and one SMAD4-lost tumor) were injected into both hind flanks of 8-week-old, NOD-SCID gamma mice (The Jackson Laboratory). In total, 1×10^5 cells were injected in each flank. The tumoroids were allowed to grow in the mice until the tumor size reached 50 mm^2. The mice were then randomized into two groups for intraperitoneal injections: 5-FU (10 mice/group) or PBS (vehicle; 6 mice/group), 40 mg/kg (and equal volume for PBS) twice per week. Mice were euthanized 7 weeks after starting the intraperitoneal injections to avoid necrosis of the tumors at necropsy. The animal studies were approved by the local Institutional Animal Care and Use Committee.

Results

SMAD4 expression in each tumor ($n = 364$) was assessed by IHC; representative staining patterns are shown in Fig. 1. Patient characteristics are shown by SMAD4 status in Table 1. Of the 364 tumors, 46 (13%) demonstrated SMAD4 loss. Those with SMAD4 loss versus SMAD4 retention did not differ significantly in gender or age. Of the SMAD4-lost tumors, 74% were either stage III or IV, whereas only 53% of SMAD4-retained tumors were stage III or IV ($P = 0.01$). All systemic chemotherapy treatments were 5-FU–based. We noted that significantly more patients with SMAD4 loss were treated with 5-FU–based chemotherapy (80%, vs. 61% of those with SMAD4 retention; $P = 0.012$).

Table 1. Clinical characteristics of discovery cohort, by SMAD4 IHC status

	SMAD4 Loss ($n = 46$) Mean (SD)	SMAD4 Retention ($n = 318$) Mean (SD)	*P*
Age	56 (15)	56 (15)	0.97
	***n* (%)**	***n* (%)**	
Sex			0.29
Male	20 (43)	165 (52)	
Female	26 (57)	153 (48)	
T-stage			0.022
T1	1 (2)	20 (6)	
T2	3 (7)	57 (18)	
T3	26 (57)	186 (59)	
T4	16 (35)	53 (17)	
N-stage			0.0088
N0	13 (28)	166 (52)	
N1	20 (43)	96 (30)	
N2	13 (28)	55 (17)	
Tumor differentiation			0.97
Poorly	1 (2)	9 (3)	
Moderately	38 (84)	262 (83)	
Well	6 (13)	43 (14)	
AJCC stage			0.011
I/II	4/8 (9/17)	50/97 (16/31)	
III/IV	22/12 (48/26)	97/74 (31/23)	
TIL score			0.031
<15	35 (78)	192 (61)	
15+	10 (22)	122 (39)	
PLA			0.0046
None	32 (71)	188 (60)	
Few	12 (27)	125 (40)	
Many	1 (2)	0 (0)	
MMR			0.024
Proficient	43 (96)	253 (82)	
Deficient	2 (4)	54 (18)	
Location			0.22
Right-sided	14 (30)	127 (40)	
Left-sided	32 (70)	191 (60)	
Chemotherapy			0.012
Yes	37 (80)	195 (61)	
No	9 (20)	123 (39)	
Recurrence			—
Yes	17 (37)	67 (22)	
None	29 (63)	249 (78)	

The majority of tumors were left-sided, but right and left colon locations did not differ significantly in frequency of SMAD4 loss (14% of left-sided tumors compared with 10% of right-sided tumors; $P = 0.22$). A total of 84 patients (23%) had recurrence of disease, and SMAD4 loss was associated with recurrence (37% of patients with SMAD4 loss vs. 22% with SMAD4 retention had recurrence, formally evaluated using survival analyses, reported later). A total of 56 tumors (15%) showed MMR protein loss. MMR loss was found in only 4% of SMAD4-lost tumors, compared with 15% of SMAD4-retained tumors ($P = 0.02$). Finally, SMAD4 loss was associated with significantly lower TIL and PLA scores in univariate analysis ($P = 0.03$; Table 1). Moreover, lower TIL and PLA scores were each associated with a significantly

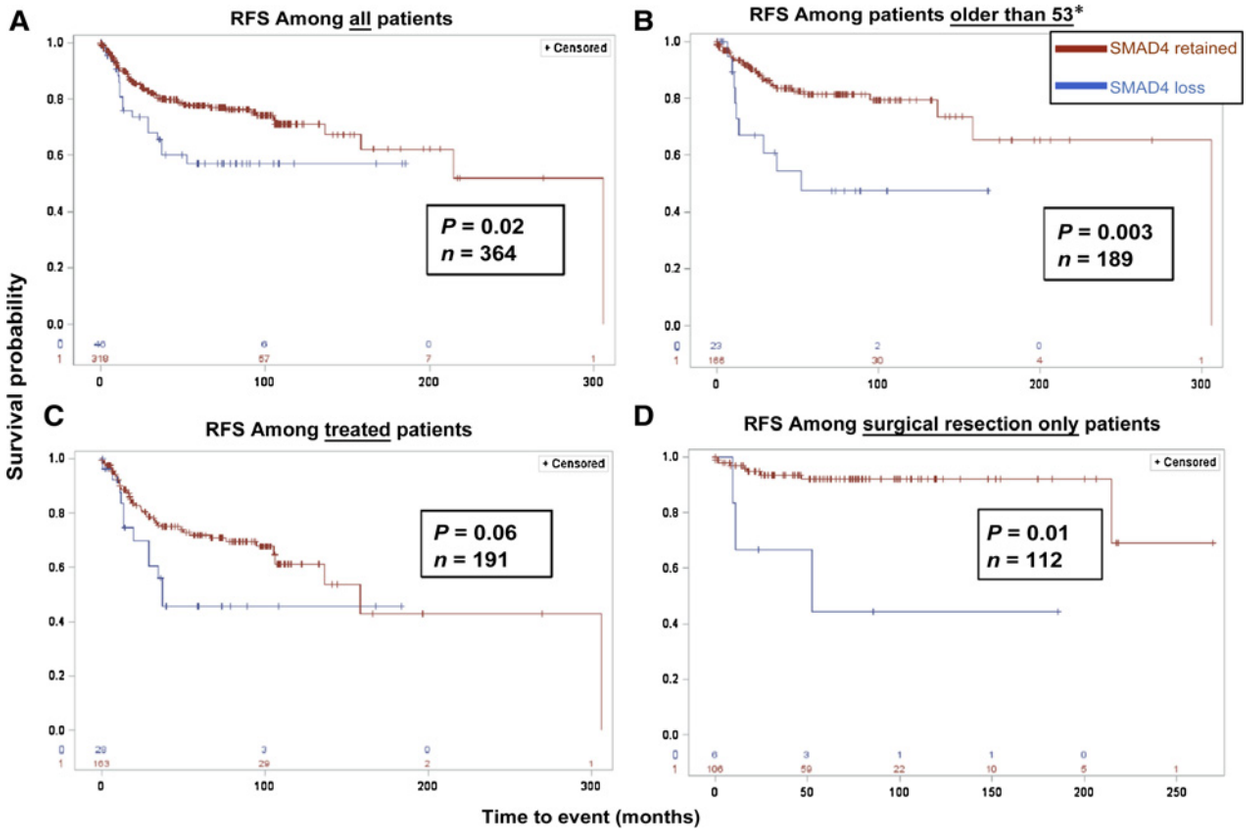

Figure 2.

Kaplan–Meier graphs with number of subjects at risk comparing RFS by SMAD4 IHC status. **A,** RFS among all patients. **B,** RFS among patients older than the median age (53 years). **C,** RFS among patients who received systemic treatment. **D,** RFS among patients who underwent surgical resection only (and received no adjuvant therapy). *, RFS did not significantly differ by SMAD4 status among patients younger than 53.

decreased RFS ($P = 0.002$ and $P = 0.01$, respectively; Supplementary Fig. S1).

Patients were followed for a median of 5.4 years (interquartile range, 2.3–8.2 years; maximum 33 years). Among all patients, RFS was significantly shorter in those with SMAD4 loss ($P = 0.02$; Fig. 2A). This finding persisted even after excluding MMR-deficient patients (Supplementary Fig. S2). In addition, a shorter RFS with SMAD4 loss was also seen among patients older than the median age of 53 ($P = 0.003$; Fig. 2B), among those who received systemic chemotherapy ($P = 0.06$; Fig. 2C), and among those who underwent surgical resection only and received no adjuvant chemotherapy ($P = 0.01$; Fig. 2D).

To adjust for the effect of confounding variables, a Cox proportional hazards model for RFS was created. Without controlling for any other variables, SMAD4 loss was associated with an increased hazard of recurrence (HR = 1.46; $P = 0.02$; Table 2) when compared with SMAD4 retention. After controlling for age at diagnosis, SMAD4 loss remained associated with an increased hazard of recurrence (HR = 1.46; $P = 0.03$; Table 2). Similarly, after controlling for tumor differentiation, SMAD4 was associated with an increased hazard of recurrence of (HR = 1.43; $P = 0.05$; Table 2). Finally, SMAD4 had near-significant associations with recurrence after controlling for stage, TIL, and PLA (respectively, HR = 1.38, 1.39, and 1.41; $P = 0.08$, 0.08, and 0.07; Table 2).

Table 2. Cox proportional hazards model for risk of recurrence associated with SMAD4 loss

SMAD4 Loss	Hazard of recurrence	*P*
Univariate	1.46	0.02
Controlled for age at diagnosis	1.46	0.03
Controlled for tumor differentiation	1.43	0.05
Controlled for stage	1.38	0.08
Controlled for TIL (<15 vs. ≥15)	1.39	0.08
Controlled for PLA	1.41	0.07
Controlled for all of the above	1.36	0.12

To further examine our findings on univariate analysis related to SMAD4 status and immune infiltrate (i.e., TIL and PLA), we compared RFS with SMAD4 and immune infiltrate status (Fig. 3). Patients with either SMAD4 or immune infiltrate loss had worse RFS than patients without either, but patients with SMAD4 loss and low TIL (<15) or SMAD4 loss and no PLA had the worst RFS ($P = 0.002$ and $P = 0.006$, respectively; Fig. 3A and B). These significant survival differences persisted even in a subgroup analysis examining only MMR-proficient patients (Supplementary Fig. S2).

The independent validation cohort ($n = 224$) found SMAD4 loss in 22% of patients ($n = 49$; Supplementary Table S2). Similar to the discovery cohort, those with SMAD4 loss versus SMAD4 retention did not differ significantly in gender or age. Of the SMAD4-lost tumors, 61% were either stage III or IV, whereas

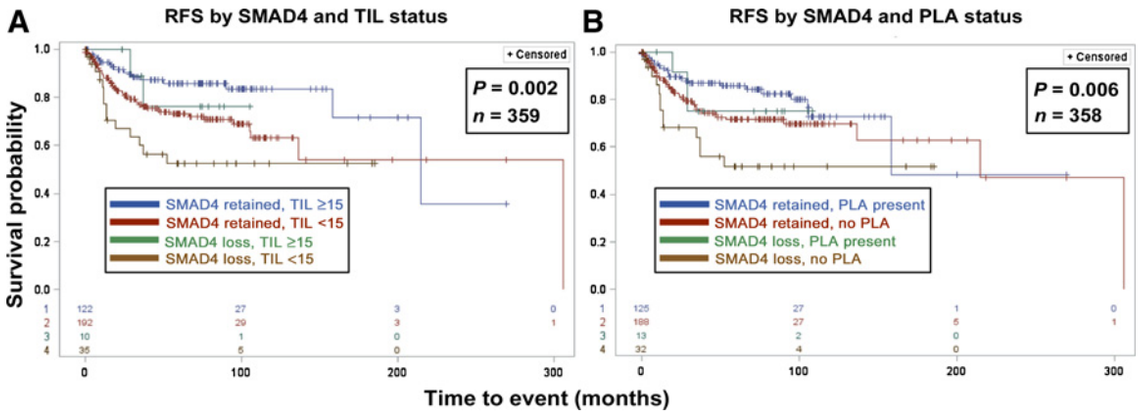

Figure 3.
Kaplan–Meier graphs with number of subjects at risk comparing RFS by SMAD4 IHC status and TIL status (**A**) or PLA status (**B**).

only 38% of SMAD4-retained tumors were stage III or IV ($P = 0.04$). Also similar to the discovery cohort, tumor location (proximal vs. distal vs. rectum) was not significantly associated with SMAD4 status. Likewise, MMR loss was found in only 4% of SMAD4-lost tumors, compared with 25% of SMAD4-retained tumors ($P = 0.001$). SMAD4 loss in the validation cohort was also associated with lower TIL ($P = 0.005$; Supplementary Table S3), as well as a trend toward decreased peritumoral lymphocytic reaction ($P = 0.08$). Finally, SMAD4 loss was associated with a trend toward worse cancer-specific survival (Supplementary Fig. S3).

Given our finding that SMAD4 correlates with survival in patients with colorectal cancer and that we noted a clinically important RFS decrease in patients with SMAD4 loss who received 5-FU–based chemotherapy (median RFS of 3.8 years vs. 13 years for those with retained SMAD4), we examined whether SMAD4 loss was, in fact, associated with resistance to 5-FU–based therapy in preclinical models. Patient-derived organoids (tumoroids) grown from patient-derived tumors and designated as SMAD4-retained and SMAD4-lost as predicted by the patient's MSK-IMPACT profile (13) and then validated by Western blot analysis (Fig. 4A) were injected into hind flanks of mice (Materials and Methods). The mice were then randomized to receive twice-weekly injections of 5-FU or vehicle (17). In the SMAD4-retained tumors, 5-FU treatment correlated with a 31% reduction in mean tumor weight ($P = 0.02$; Fig. 4B). In the SMAD4-lost tumors, however, 5-FU treatment had no significant effect on tumor weight ($P = 0.76$; Fig. 4C). To extend these findings, we examined how altering SMAD4 levels affects chemosensitivity of patient-derived tumoroids and a colorectal cancer cell line model. Specifically, in SMAD4-retained tumoroids derived from two additional patients, we observed that SMAD4 knockdown induced significant resistance to both 5-FU and FOLFOX (Fig. 4D and E). Conversely, in a SMAD4-deficient cell line (SW480), we observed that SMAD4 restoration induced sensitivity to 5-FU and FOLFOX (Fig. 4F and G).

Discussion

In our discovery cohort, a single-center study investigating SMAD4 loss in 364 patients with colorectal cancer at a tertiary cancer care center, we found that loss of SMAD4 expression by IHC was associated with significantly worse RFS. This association persisted throughout sensitivity analyses, which examined RFS among MMR-proficient patients, as well as among patients who received resection only. SMAD4 loss by IHC, as evidenced in 13% of the tumors, was associated with high tumor and nodal stage and with adjuvant therapy use. It was also associated with lower TIL and PLA scores. This finding of an association between SMAD4 loss and decreased immune infiltrate was confirmed in an independent, validation cohort. To our knowledge, we are the first to report this association between SMAD4 loss and the immune infiltrate as measured by TIL and PLA in colorectal cancer in both univariate and survival analyses; the occurrence of SMAD4 loss and TIL/PLA loss had the greatest impact on RFS. These novel findings, found both in the discovery and validation cohorts, shed light on a putative relationship between SMAD4 and immune evasion and underscore the clinical importance of SMAD4 as a potential prognostic biomarker in patients with colorectal cancer.

SMAD4 loss, in a study of 241 patients using TMAs, was associated with worse overall survival (OS) and RFS (6). In a subset of these patients receiving capecitabine, those with SMAD4 loss also had significantly worse RFS and OS. In a recent meta-analysis, SMAD4 loss correlated with worse cancer-specific survival, disease-free survival, and OS in pooled multivariate analyses (18). These studies suggest that the association of SMAD4 loss with worse outcomes is mediated through chemoresistance to 5-FU (19). Some of these studies, however, have suffered from small sample size and short median length of follow-up (6, 18). Our finding that SMAD4 loss is associated with worse RFS reinforces and validates these studies (6, 18). Our data further build on the recent findings by Yan and colleagues, who found that SMAD4 loss was associated with shorter survival in patients with stage III colorectal cancer (7). In addition, we found that SMAD4 loss was associated with strikingly worse RFS in the subset of patients who received 5-FU–based therapies (3.8 years median RFS for patients with SMAD4 loss compared with 13 years for those with retained SMAD4 expression), adding to the evidence that SMAD4 loss may promote chemoresistance to 5-FU–based treatments. SMAD4 loss may also correlate with resistance to FOLFIRI (leucovorin, fluorouracil, and irinotecan), on the basis of recent data from the Pan European Trial Adjuvant Colon Cancer (PETACC-3) trials (7, 20). It remains unknown whether patients with colorectal cancer with SMAD4 loss are good candidates for

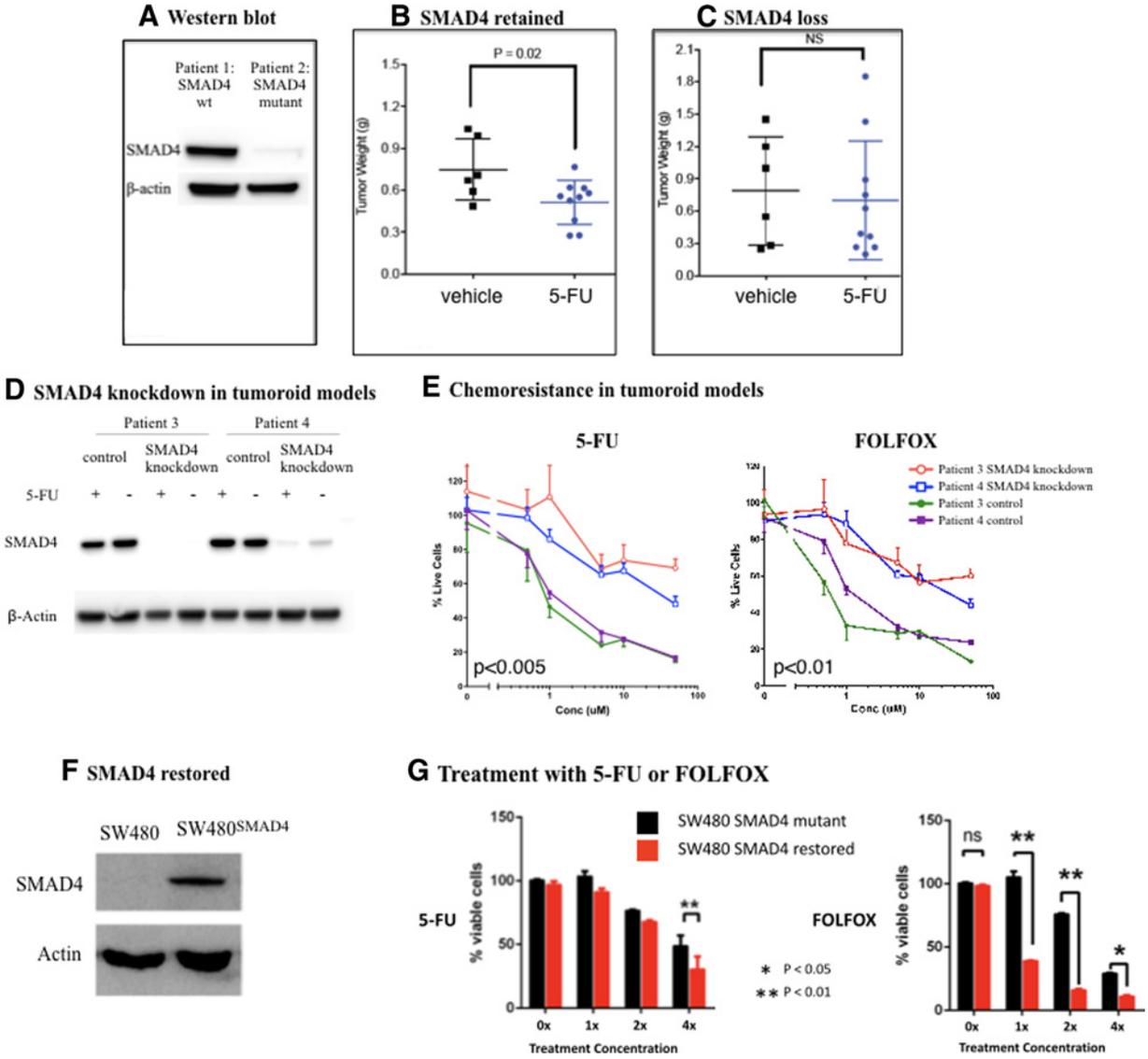

Figure 4.

SMAD4 and the 5-FU sensitivity of colorectal cancer xenografts, tumoroids, and cell lines. **A,** Western blot analysis validating SMAD4 status of patient-derived tumor cells. The blots analyzed tumoroids grown from cells from a SMAD4-retained tumor (patient 1; SMAD4 wild-type) and a SMAD4-lost tumor (patient 2; SMAD4 mutant). **B,** Weight of tumors grown in mice from cells from the patient 1 tumoroid (retained SMAD4) after treatment with 5-FU or vehicle. **C,** Weight of tumors grown in mice from cells from the patient 2 tumoroid (SMAD4 loss) after treatment with 5-FU or vehicle. The central horizontal lines represent the mean, and the whiskers represent the SD. NS, not significant. **D,** Western blot analysis of two additional patient-derived tumoroids (patients 3 and 4) transfected with a control sgRNA lentiviral vector or sgRNA lentiviral against SMAD4, showing that SMAD4 was knocked down by the SMAD4-targeting sgRNA. **E,** Sensitivity of tumoroids with and without SMAD4 knockdown to 5-FU (left) and FOLFOX (right). Data shown are the averages of biological duplicate samples; error bars display SE. The differences in the control group and SMAD4-knockdown group were assessed using ANOVA. **F,** Western blot confirming the expected lack of SMAD4 in SW480 colorectal cancer cells (which are SMAD4 mutant) and restoration of SMAD4 in $SW480^{SMAD4}$ cells (which were transduced with *SMAD4*). **G,** Cell viability of SW480 and $SW480^{SMAD4}$ cells after treatment with increasing concentration (1×, 2×, and 4×) of 5-FU (left) or FOLFOX (right; the data represent mean ± SD; *, $P < 0.05$; **, $P < 0.01$ vs. control).

irinotecan or oxaliplatin chemotherapy, or whether alternative therapies should be explored in this subset of patients. Of note, however, SMAD4 loss also correlated with worse RFS among patients who were treated with surgical resection only. The worse RFS among surgical resection–only patients may simply indicate a worse natural history for patients with SMAD4-loss colorectal cancer. The worse RFS among treated patients, coupled with our *in vivo* data suggesting 5-FU resistance among SMAD4-loss tumors, may indicate that on top of the unfavorable natural history of SMAD4 loss, there is an additional, SMAD4-mediated chemoresistance contributing to worse RFS.

This study, in both the discovery and validation cohorts, confirms the previously identified association between SMAD4 expression and MMR status: tumors with SMAD4 loss are mostly

MMR proficient (7). In addition, we found notable associations in two independent cohorts of SMAD4 loss with significantly fewer TIL and PLA. RFS was significantly worse among patients with either SMAD4 loss or immune infiltrate loss, and the worst RFS occurred in those with loss of both. This association persisted in a sensitivity analysis controlling for MMR deficiency. Although the SMAD4-retained cohort had higher rates of MMR deficiency (likely increasing their tumor immune infiltrate), differences in survival persisted in a sensitivity analysis of just MMR-proficient tumors (21). Furthermore, our study combines the clinical association of SMAD4 loss and shorter RFS with an *in vivo* model of SMAD4-mediated chemoresistance. Thus, our work integrates data from clinical survival outcomes and patient-derived *in vivo* models, further strengthening the association of chemoresistance to 5-FU with SMAD4 loss and provides a potential model to elucidate the mechanism behind this resistance. Although this patient-derived model did not allow for corroboration of the association of SMAD4 loss with immune infiltrate, it is a promising avenue for hypothesis generation and future study.

A strength of this study is the replication of many of the initial findings in an independent, validation cohort. Although the association between SMAD4 loss and worse survival did not reach statistical significance in the independent cohort ($P = 0.1$), this was most likely due to reduced power ($n = 224$). The ability of another cohort to replicate the findings in the discovery cohort, especially while using a different SMAD4 antibody, increases the likelihood of the generalizability of our findings and provides new insights to potential biology apt for further exploration.

Our study is limited by the inherent issues of retrospective studies, including potential selection bias. In addition, as with any IHC assay, the assessment of staining is subject to intra- and interobserver variability. We attempted to account for this weakness in two ways. First, we set standards for SMAD4 loss versus retention in both cohorts based on review of the literature (6, 12) and assessment of the stains. Second, dedicated GI pathologists scored all the cases independently, and disagreements were reconciled to reach a consensus. A final limitation is that, given the small number of SMAD4 loss cases ($n = 46$), we could not use an exhaustive, multivariable Cox proportional hazards model due to the risk of overfitting (22). Nonetheless, we performed a stepwise, forward selection model to control for selected, clinically relevant variables. When adjusting for stage, the association between SMAD4 loss and RFS only trended toward significance, likely a result of the small number of cases. Larger cohorts with more cases of SMAD4 loss are best suited to reproduce this finding.

In summary, we found loss of SMAD4 by IHC in 13%–22% of two independent cohorts of stage I–IV colorectal cancers. SMAD4 loss correlated with higher tumor and nodal stage, with adjuvant therapy use, and with lower TIL and PLA scores. In addition, SMAD4 loss was associated with worse RFS, even among the resection-only group. In a Cox regression model, SMAD4 loss is associated with a significantly increased hazard of recurrence alone and when controlling for either age at diagnosis or tumor differentiation. When stratified by both SMAD4 and immune infiltrate status, SMAD4 loss and low immune infiltrate was associated with the worst RFS. This finding persisted even after excluding MMR-deficient patients. Furthermore, we corroborated our clinical chemoresistance findings in patient-derived cell lines, tumoroids, and xenografts, demonstrating that loss of SMAD4 confers resistance to 5-FU. These data further strengthen the rationale for using SMAD4 expression as a marker to aid in clinical risk assessment, as well as further suggest a role for SMAD4 in the immune environment in colorectal cancer pathogenesis.

Disclosure of Potential Conflicts of Interest

J.G. Guillem is a consultant/advisory board member for Roche China. A. Cercek reports receiving other commercial research support from Amgen, AbbVie, and Seattle Genetics, and is a consultant/advisory board member for Bayer. C.L. Sawyers is a consultant/advisory board member for Novartis, Agios, Beigene, Blueprint, Column Group, Nextech, Housey Pharma, Foghorn, KSQ, PMV, ORIC, and Petra. J. Garcia-Aguilar reports receiving speakers bureau honoraria from Intuitive Surgical, Johnson and Johnson, and Medtronic. M. Giannakis is a consultant/advisory board member for AstraZeneca. J.J. Smith reports other remuneration from Intuitive Surgical. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The funding source had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, and approval of the manuscript; and decision to submit the manuscript for publication. The authors assume full responsibility for analyses and interpretation of these data.

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