SMAD4 Levels and Response to 5-Fluorouracil in Colorectal Cancer

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Abstract
We have recently reported that low tumor levels of SMAD4, a key mediator of transforming growth factor-β superfamily signaling, can predict the probability of recurrence in patients with Dukes C colorectal cancer who had surgery as the only form of treatment. However, standard treatment for Dukes C colorectal cancer patients currently involves the administration of 5-fluorouracil (5-FU)–based adjuvant chemotherapy after surgery. Approximately 30% to 40% of these patients present with recurrence and die within 5 years, and there is great need for markers capable of predicting poor prognosis after the combined surgery/adjuvant treatment. In this study, we evaluate the prognostic value of SMAD4 in patients treated with surgery and 5-FU-based adjuvant therapy. We used immunohistochemistry and quantitative real-time reverse transcription-PCR to measure the levels of SMAD4 protein and mRNA expression in the primary tumors and a number of lymph node metastases from a series of 75 Dukes C colorectal cancer patients with at least 6 years of follow-up. Patients with tumors expressing low levels of SMAD4 protein or mRNA showed significantly shortened disease-free and overall survival than patients with high tumor levels of SMAD4. The median survival of patients with low SMAD4 protein or mRNA tumor levels was 1.4 and 1.2 years, respectively, whereas patients with high SMAD4 tumor level had a median survival of >9.3 years. In addition, the protein and mRNA levels of SMAD4 in lymph node metastases was significantly lower than in primary tumors (P = 0.006). In contrast, allelic imbalance in chromosome 18q21 was of no prognostic significance in these patients. In conclusion, low SMAD4 tumor levels identified a subset of patients with poor prognosis following surgery and 5-FU-based adjuvant therapy; therefore, these patients could be good candidates to receive combined treatment with additional chemotherapeutic agents such as CPT-11 and/or oxaliplatin.

Approximately 50% of the patients diagnosed with Dukes C colorectal cancer have disease recurrence and die within 5 years of surgical removal of their primary tumor. Given the relatively high risk of recurrence of these patients, 5-fluorouracil (5-FU)–based adjuvant chemotherapy is routinely given to the great majority of patients with Dukes C colorectal cancer. Although this aggressive chemotherapeutic treatment prevents disease recurrence in 10% to 20% of the patients, ~30% to 40% of the patients will not respond to this treatment and will succumb to their disease (1, 2). Patients unlikely to respond to the standard 5-FU-based adjuvant therapy are good candidates to receive additional treatment. The platinum compound oxaliplatin and the camptothecin derivative CPT-11 have been shown to be effective chemotherapeutic agents that can be used alone or in combination with 5-FU (3–6). The availability of these newer chemotherapeutic agents in addition to more experimental treatment options, such as cyclooxygenase-2 or epidermal growth factor receptor inhibitors (7, 8), highlights the need for markers capable of identifying the subset of patients that is unlikely to respond to the standard 5-FU-based adjuvant treatment.

Although a number of genetic markers of response to treatment for patients with locally advanced colorectal cancer have been investigated, it is currently not possible to accurately predict the probability of recurrence of Dukes C patients (9–11). Losses of genetic material in the long arm of chromosome 18 are known to be associated with poor survival of Dukes B colorectal cancer, although the prognostic significance for lymph node–positive Dukes C patients remains controversial (12–17). These frequent deletions in 18q are believed to be indicative of the presence of one or more tumor suppressor genes in this region (18, 19). SMAD4 is a key mediator of
transforming growth factor-β superfamily signaling that regulates cell growth and apoptosis and is located in 18q21 (20–22). SMAD4 mutations have been linked to juvenile polyposis, a colorectal cancer predisposition syndrome (23), and frequent point mutations have been observed in sporadic colorectal tumors (24–26), suggesting that this gene is an important target for the 18q deletions. We have recently shown that reduced tumor levels of SMAD4 protein are associated with significantly poorer disease-free and overall survival after potentially curative surgery in Dukes C colorectal cancer patients (24). However, standard treatment for Dukes C colorectal cancer patients currently incorporates 5-FU-based adjuvant treatment in addition to surgery, and the potential of SMAD4 levels as a marker of response to the combined surgery/adjuvant treatment has not been investigated.

Here we demonstrate that low levels of SMAD4 protein in the primary tumors of Dukes C colorectal cancer patients are associated with significantly shorter overall and disease-free survival in patients that had surgery followed by 5-FU-based adjuvant therapy. Moreover, we show that tumor SMAD4 mRNA levels can also be used to predict treatment response in Dukes C patients, and that lymph node metastases have lower levels of SMAD4 mRNA and protein that primary tumors. In contrast, allelic imbalance in chromosome 18q21 was of no prognostic significance in these patients.

Materials and Methods

Assessment of SMAD4 protein levels: Patient samples. Formalin-fixed, paraffin-embedded samples from 75 primary tumors and 16 lymph node metastases from Dukes C colorectal cancer patients were collected at collaborating institutions in Southern Finland from 1994 to 1998. Informed consent for genetic analysis of the tumor sample was obtained from each patient according to the Human Investigations and Ethical Committee–approved research proposal. All patients received 5-FU-based adjuvant chemotherapy after potentially curative surgery. Complete follow up was available for at least 6 years (mean follow-up, 8.7 years). The clinical features of the 75 patients entered in the study are shown in Table 1. Although tumor-node-metastasis staging was not routinely used at the time of sample collection in the collaborating institutions, information recorded in the pathology reports allowed tumor-node-metastasis staging of 47 of the 75 (63%) patients in the study (Table 1).

Tissue microarray. After histologic examination of H&E-stained sections of formalin-fixed, paraffin-embedded tumor samples by an experienced pathologist (R.S.), areas containing a high proportion of tumor cells were selected from all 75 patients. Triplet 0.6-mm cores of tumor samples from every patient were arrayed in a fresh paraffin block using a Beecher Instruments tissue arrayer (Beecher Instruments, Silver Spring, MD).

Immunohistochemistry. A SMAD4 monoclonal antibody raised against a peptide corresponding to amino acids 1 to 552 representing full-length SMAD4 of human origin was used (1:1,000 dilution; SMAD4 clone B8, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specificity of this antibody has been previously tested in formalin-fixed paraffin-embedded samples (24, 27, 28). Unstained 4-μm sections from the tissue microarray were mounted on slides coated with 3-aminopropyl-triethoxysilane (Sigma, St. Louis, MO). Sections were deparaffinized in xylene and rehydrated through a graded alcohol series and distilled water. The sections were treated with 3% H2O2 solution for 10 minutes to block endogenous peroxidase activity. Sections were then treated in a microwave oven in 10 mmol/L sodium citrate buffer (pH 6) for 5 minutes at 800 W and 10 minutes at 450 W. For immunohistochemical analysis, the commercial PowerVision Poly-horseradish peroxidase immunohistochemistry detection kit was used (ImmunoVision Technologies, Brisbane, CA) as previously reported (24). SMAD4 antigen expression was independently evaluated in the triplicate tumor samples from the 75 colorectal cancer patients by two researchers (D.A. and R.S.) blinded from the clinical data. A semiquantitative scale from 0 to 4 was used to assess the intensity of the staining. Samples scored as 0 showed no SMAD4 staining and samples with the highest staining intensity were scored as 4. The average score of replicate samples was used in subsequent analyses.

A systematic approach was used to identify the optimal threshold to divide the samples into a group with high SMAD4 expression and a group with low SMAD4 protein level. Mean survival in both groups, hazard ratio, and log-rank P values were calculated for every possible grouping resulting from increasing the number of patients allocated to the low SMAD4 group from 1 to 75, starting with the patient with the lowest SMAD4 tumor level (see Supplementary Table 1).

Western blotting. Cells lysates from three cell lines (DLD1, HCT116, and SW620) and three primary fresh frozen colorectal tumors were extracted using radioimmunoprecipitation assay buffer as previously described (29, 30). Ninety-microgram aliquots were loaded onto a 10% polyacrylamide gel, and fractionated proteins were transferred to a nitrocellulose membrane. Immunostaining with

| Table 1. Clinical features of the 75 patients in the series used in this study |
|---------------------------|-----------------------------|-----------------------------|
|                          | Total Low SMAD4 High SMAD4 P |
| Sex, n (%)               |                             |                             |
| Female 36                | 6 (60)                      | 30 (46.1)                   | 1.0* |
| Male 39                  | 4 (40)                      | 35 (53.9)                   |     |
| Age (y) 59 ± 11.6        | 61 ± 8.1                    | 58.7 ± 12.1                 | 0.7* |
| Tumor site, n (%)        |                             |                             |
| Colon 50                 | 6 (60)                      | 44 (67.7)                   | 0.7* |
| Rectum 25                | 4 (40)                      | 21 (32.3)                   |     |
| Degree of differentiation of tumor, n (%) | 8 (12.3) | 0.25* |
| Good 9                   | 1 (10)                      | 8 (12.3)                    |     |
| Moderate 56              | 6 (60)                      | 50 (76.9)                   |     |
| Poor 10                  | 3 (30)                      | 7 (10.8)                    |     |
| Tumor-node-metastasis stage, n (%) | 8 (12.3) | 0.73 |
| T2N1 4                   | 0 (0.0)                     | 4 (9.8)                     |     |
| T3N1 31                  | 4 (66.7)                    | 27 (65.9)                   |     |
| T3N2 7                   | 1 (16.7)                    | 6 (14.6)                    |     |
| T4N1 3                   | 1 (16.7)                    | 2 (4.9)                     |     |
| T4N2 2                   | 0 (0.0)                     | 2 (4.9)                     |     |
| 5-y overall survival, n (%) | 38 (58.5) | 0.019* |
| Alive 40                 | 2 (20)                      | 38 (58.5)                   |     |
| Dead 35                  | 8 (80)                      | 27 (41.5)                   |     |

*P values calculated using Fisher’s exact test for the comparison of high and low SMAD4.

†P values calculated using Mann-Whitney test for the comparison of high and low SMAD4.

‡P values calculated using Chi-square for the comparison of high and low SMAD4.
anti-SMAD4 antibody (1:1,000 dilution; SMAD4 clone B8, Santa Cruz Biotechnology) was carried out as previously described (29, 30). Immunostaining with an anti-β-actin antibody (1:2,000 dilution; Actin (Ab-1), Oncogene Research Products, San Diego, CA) was used as a loading control.

Assessment of mRNA SMAD4 levels: RNA extraction. Four-micrometer sections from all blocks available from the 75 tumors and 16 lymph node metastases entered in this study were cut and stained with H&E to evaluate sample tumor content. A total of 28 primary tumor samples and five lymph node metastases were found to contain at least 50% of tumor cells and RNA was extracted from these samples. Six 10-μm sections were cut from the selected blocks and total RNA extracted as previously described (31). Briefly, the sections were added to 850 μL of denaturing solution (4 mol/L guanidinium isothiocyanate/0.25 mol/L sodium citrate/0.5% sarcosyl/0.1 mol/L 2-mercaptoethanol) and 250 μL of proteinase K solution (20 mg/mL in H2O). Samples were then incubated overnight at 55°C in a thermoshaker. The resulting lysate was phenol/chloroform extracted and the RNA in the aqueous phase was isopropanol precipitated and resuspended in 50 μL of water.

Quantitative real-time reverse transcription-PCR. SMAD4 mRNA levels were quantified using real-time reverse transcription-PCR. Aliquots of RNA (100 ng) were reverse transcribed using Superscript II according to manufacturer’s recommendations (Invitrogen, Carlsbad, CA). Five microliters of the undiluted reverse transcriptase reaction were used to PCR amplify SMAD4 using the following primers and Taqman probe: forward, 5'AAAACCCGGCCTATCTTGACGAC-3'; reverse, 5'AGGCC-CAGTAAATGCCTCGGGA-3'; probe, 5'FAM-ACCCGCCATGTCGCCCCC-TAMRA-3'. Both primers and the Taqman probe were at a final concentration of 200 nmol/L in the final reaction, which was done using Taqman Universal PCR Master Mix in a GeneAmp 5700 Sequence Detection System (both from Applied Biosystems, Branchburg, NJ).

Relative levels of SMAD4 were quantified using the ΔΔCt method as previously described (30, 32). The optimal threshold to allocate tumors to the high or low SMAD4 group was calculated as described for the SMAD4 protein levels.

Assessment of allelic imbalance. Genomic DNA was extracted from fresh frozen tumor and normal mucosa samples from the 75 patients entered in this study. Toluidine blue–stained frozen sections were evaluated histologically by a pathologist before DNA extraction to document the proportion of tumor tissue as previously reported (33–35). The percentage of tumor cells was at least 50% in 69 of the 75 samples (92%). Two polymorphic microsatellite markers in 18q21 (33–35). The percentage of tumor cells was at least 50% in 69 of the 75 patients entered in this study. Toluidine blue–stained frozen sections were used to PCR amplify SMAD4 using the following primers and Taqman probe: forward, 5'AAAACCCGGCCTATCTTGACGAC-3'; reverse, 5'AGGCC-CAGTAAATGCCTCGGGA-3'; probe, 5'FAM-ACCCGCCATGTCGCCCCC-TAMRA-3'. Both primers and the Taqman probe were at a final concentration of 200 nmol/L in the final reaction, which was done using Taqman Universal PCR Master Mix in a GeneAmp 5700 Sequence Detection System (both from Applied Biosystems, Branchburg, NJ).

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ment and that is at high risk of recurrence (5-year recurrence rate of 80%).

**Low SMAD4 mRNA levels are associated with poor prognosis after 5-fluorouracil-based adjuvant therapy.** We next wanted to investigate whether the levels of SMAD4 mRNA could be used as a prognostic marker in Dukes C colorectal cancer patients that received 5-FU-based adjuvant therapy. Because whole tissue lysates are used for assessment of relative mRNA SMAD4 levels, it is important to select samples that contain a significant number of tumor cells. For this purpose, we assessed the proportion of tumor cells in all the formalin-fixed, paraffin-embedded tumor samples available from the 75 primary tumors and 16 lymph node metastases included in this study. Total RNA was extracted from paraffin-embedded samples of the 28 primary tumor samples and five lymph node metastases that contained at least 50% of tumor cells. The relative SMAD4 mRNA levels were assessed using quantitative real-time reverse transcription-PCR. To identify the threshold of SMAD4 mRNA tumor levels that best separates patients with good and bad response to treatment, we systematically compared the survival curves obtained with every possible grouping as we did for the protein levels (Supplementary Table 2). As observed for the SMAD4 protein levels, patients with tumors expressing low levels of SMAD4 mRNA showed shorter disease-free survival for every grouping with more than four patients compared with patients in the high SMAD4 group, irrespectively of the cutoff selected to allocate patients to the high or low SMAD4 group (Supplementary Table 2). These differences in disease-free survival were statistically significant ($P < 0.05$, log-rank test) for eight of these groupings. A similar trend was observed when comparing the overall survival of patients with high and low SMAD4 mRNA tumor levels, and when 7 of the 28 (25%) patients are considered to have low SMAD4 mRNA tumor levels, the 5-year disease-free rate for patients with low SMAD4 tumor levels was 30%, whereas 80% of the patients whose tumors expressed high SMAD4 mRNA survived >5 years from initial surgery (Fig. 3; log-rank test, $P = 0.003$ and $P = 0.01$ for disease-free and overall survival, respectively). There were no association between SMAD4 mRNA levels and other clinicopathologic variables such as patient sex, age, tumor location (colon/rectum), histologic grade, and tumor-node-metastasis stage (data not shown). On a multivariate analysis, SMAD4 mRNA level was a strong predictor of shorter disease-free and overall survival ($P = 0.014$ and $P = 0.008$, respectively, Cox proportional hazards model). These results are in good agreement with the survival differences observed in patients with different SMAD4 protein levels, and a significant correlation was observed between mRNA and protein levels of SMAD4 in these 28 patients (Spearman $r = 0.4$, $P = 0.04$). Moreover, as observed for the SMAD4 protein levels, metastatic lesions were also found to have significantly lower SMAD4 mRNA levels than primary tumors (Student’s $t$ test, $P = 0.006$), further suggesting that reduced SMAD4 levels are associated with disseminated disease, and in good agreement with the notion that low SMAD4 is a marker of poor prognosis.

![Fig. 2. Overall survival (A) and disease-free survival (B) according to SMAD4 protein levels in Dukes C colorectal cancer patients that had potentially curative surgery and 5-FU-based adjuvant chemotherapy (Kaplan-Meier plots). Log-rank P values.](image1)

![Fig. 3. Overall survival (A) and disease-free survival (B) according to SMAD4 mRNA levels in Dukes C colorectal cancer patients that had potentially curative surgery and 5-FU-based adjuvant chemotherapy (Kaplan-Meier plots). Log-rank P values.](image2)
Allelic imbalance in chromosome 18q. SMAD4 is located on chromosome 18q21. Although allelic imbalance in 18q has been shown to be associated with poorer prognosis in patients with Dukes B colorectal cancer (13, 15, 36), the accuracy of this genetic marker of prognosis in Dukes C patients remains controversial (12–17, 37). Therefore, we decided to investigate the prognostic significance of genetic abnormalities in chromosome 18q in this series of 75 Dukes C colorectal cancer patients. Two polymorphic microsatellite markers (D18S1110 and D18S1156) in 18q21 were used to assess the presence of allelic imbalance in this region. Thirty-two of the 75 tumors (43%) were either homozygous for both markers or did not amplify in the PCR reactions. Of the remaining 43 samples, 21 (49%) showed allelic imbalance in at least one of these two markers, whereas the remaining 22 (51%) showed no evidence of allelic imbalance. In agreement with previous investigations (12, 15, 17, 24, 37), our results showed no difference in the overall survival rate between patients with or without allelic imbalance in 18q21 (Fig. 4; log-rank test, $P = 0.8$ and $P = 0.6$, respectively), and there were no significant differences in the level of SMAD4 protein and mRNA in tumors with and without allelic imbalance in 18q (Student’s $t$ test, $P = 0.8$ and $P = 0.7$, respectively).

Discussion

We have recently reported that low SMAD4 tumor protein level is a marker of poor prognosis for Dukes C colorectal cancer patients that had surgery as the only form of treatment (24). However, routine clinical management of Dukes C colorectal cancer patients currently involves administration of 5-FU-based adjuvant chemotherapy in addition to surgery, and the prognostic value of SMAD4 in this setting has not been investigated. Here we show that low levels of tumor SMAD4 protein can identify a subset of Dukes C colorectal cancer patients with high probability of recurrence following potentially curative surgery and 5-FU-based adjuvant chemotherapy. In addition, we report here for the first time that SMAD4 mRNA levels assessed by quantitative realtime reverse transcription-PCR from formalin-fixed, paraffin-embedded tumor samples closely correlate with tumor protein levels and that low level of SMAD4 mRNA expression in the primary tumor of Dukes C patients is a marker of poor prognosis.

SMAD4 is a key transducer of transforming growth factor-$
\beta$ superfamily signaling. Transforming growth factor-$
\beta$ regulates proliferation and differentiation of normal colonic epithelium, and transforming growth factor-$
\beta$ signaling inactivation is one of the hallmarks of colorectal carcinogenesis (38–40). SMAD4 is located in the long arm of chromosome 18, one of the genomic regions most commonly deleted in colorectal tumors (18, 19). Recently, this transcription factor has received substantial attention as a tumor suppressor gene targeted by these deletions, because inherited mutations in this gene have been associated with colorectal cancer predisposition (41). Inactivation of tumor suppressor genes is commonly associated with tumor progression and poor patient prognosis. This is consistent with earlier reports showing that SMAD4 mutations are more common in late-stage colorectal tumors (13, 14, 26). In good agreement, here we show that low levels of tumor SMAD4 mRNA or protein are associated with significantly poorer patient prognosis and that lymph node metastases express lower levels of SMAD4 than primary tumors. Importantly, low level of SMAD4 mRNA or protein expression in colorectal tumors is a better predictor of the probability of recurrence than loss of heterozygosity in chromosome 18q for Dukes C patients that received 5-FU adjuvant chemotherapy.

5-FU has been the main chemotherapeutic agent used to fight colorectal cancer for over four decades. However, additional agents such as the platinum compound oxaliplatin and topoisomerase-I inhibitors such as CPT-11, have recently entered the clinical arena. Low tumor levels of SMAD4 could be used to identify poor prognosis Dukes C patients with tumors that are otherwise histologically indistinguishable. For example, the level of SMAD4 mRNA can discriminate between Dukes C patients with a 5-year probability of recurrence of 30% (high SAMD4) and 80% (low SAMD4) after 5-FU-based adjuvant chemotherapy (Fig. 3). Patients in the latter group could therefore be good candidates to receive a more aggressive treatment in an attempt to improve their probability of long term survival. Additional investigations will be necessary to assess the potential benefit of these patients from the combined treatment with 5-FU and/or CPT-11/oxaliplatin.

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