More than 50% of patients with Dukes C colorectal cancer have disease recurrence and die within 5 years after surgical removal of their primary tumor. It is currently not possible to distinguish patients with good and bad prognosis. **SMAD4** is an important tumor suppressor gene that mediates transforming growth factor-β superfamily signaling and is located in chromosome 18q21, a region with frequent genetic losses in these tumors. Allelic imbalance in 18q has been linked to poor prognosis in a subset of colorectal cancer patients. Therefore, we generated a tissue microarray containing triplicate tumor samples from 86 Dukes C patients and used immunohistochemistry to assess the relative expression level of **SMAD4** and its value as a prognostic marker. In addition, **SMAD4** was screened for mutations and two polymorphic microsatellite markers were used to assess the presence of allelic imbalance in these tumors. Patients with tumors expressing high **SMAD4** levels had significantly better overall (\(P < 0.025\)) and disease-free (\(P < 0.013\)) survival than patients with low levels. This identifies **SMAD4** as a prognostic marker for Dukes C colorectal cancer. Although all tumors with absent **SMAD4** staining showed allelic imbalance in 18q21, tumors with 18q21 allelic imbalance as a group showed no difference in **SMAD4** levels compared with tumors without allelic imbalance, suggesting that additional mechanisms of **SMAD4** down-regulation exist. In addition, although **SMAD4** mutations were found in five tumors, they were not associated with shorter survival. In conclusion, the level of expression of **SMAD4** was found to be a more sensitive marker than 18q21 allelic imbalance and **SMAD4** mutations, which were of no prognostic significance for these patients.
epigenetic mechanisms of inactivation, such as promoter hypermethylation, thus potentially increasing the accuracy of 18q changes as a prognostic marker. Second, this approach could afford significant insight into the identity of the genes targeted by these often large deletions in the long arm of chromosome 18. And third, such an approach would be more amenable to routine clinical use.

In this study, we used a tissue microarray–based immunohistochemical assay to measure the level of expression of SMAD4 in 86 tumor samples from Dukes C colorectal cancer patients that did not receive adjuvant chemotherapy and showed its potential as a prognostic factor for these patients. Moreover, the accuracy of SMAD4 levels to predict prognosis was found to be superior to that obtained through analysis of 18q21 allelic imbalance or SMAD4 mutation screening in the same patients.

Materials and Methods

Patient samples. Formalin-fixed, paraffin-embedded, and fresh-frozen samples from 86 cases of colorectal cancer were collected at collaborating institutions in Southern Finland from 1993 to 1997. Complete follow-up was available for at least 6 years. To maximize the statistical power to address the specific aim of this study, only Dukes C patients that had potentially curative surgery and did not receive adjuvant chemotherapy were selected. The clinical features of the 86 patients entered in the study are shown in Tables 1 and 2.

Tissue microarray. After histologic examination of H&E-stained sections of formalin-fixed, paraffin-embedded tumor samples by an experienced pathologist (R. Salovaara), areas containing a high proportion of tumor cells were selected from all 86 patients. Triplicate 0.6 mm cores from tumor samples from every patient were arrayed in 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 (SMAD4 B-8, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specificity of this antibody has been previously tested in formalin-fixed paraffin-embedded samples (24, 25).

Unstained 4 μm sections from the tissue microarray were mounted on slides coated with 3-aminopropyl-triethoxy-silane (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-HRP IHC detection kit was used (ImmuNoVision Technologies, Brisbane, CA). After a 10-minute incubation with Preantibody Blocking Solution, slides were incubated with an horseradish peroxidase–conjugated secondary antibody (O. Kindler GmbH & Co., Freiburg, Germany). SMAD4 antigen cleared in xylene, and mounted with Eukitt mounting media (ImmunoVision Technologies, Brisbane, CA). After a 10-minute incubation with post-antibody blocking solution, slides were incubated for 2 hours with a 1:1,000 dilution of the primary antibody. Sections were then washed three times in TBS and following a 20-minute incubation with post-antibody blocking solution, slides were incubated with a horseradish peroxidase–conjugated secondary antibody for 30 minutes at room temperature. All incubations were done in a humidified chamber at room temperature. Staining was visualized using a 3,3′-diaminobenzidine solution for 1 minute at room temperature, and sections were counterstained in Mayer’s hematoxylin, rinsed in water, dehydrated through a series of ethanol solutions, cleared in xylene, and mounted with Eukitt mounting medium (O. Kindler GmbH & Co., Freiburg, Germany). SMAD4 antigen expression was independently evaluated in the triplicate tumor samples from the 86 CRC patients by two researchers (D. Arango and R. Salovaara) blinded from the clinical data. A semiquantitative scale from 0 to 4 was used to measure 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.

Assessment of allelic imbalance. Genomic DNA was extracted from fresh frozen tumor and normal mucosa samples from the 86 patients

| Table 1. Clinical features of 86 patients whose colorectal tumors were evaluated for SMAD4 levels |
|---------------------------------|----------------|----------------|-------|-----|
|                                | Low SMAD4, n (%) | High SMAD4, n (%) | P*   |
| Sex                            |                |                 |      |    |
| Female                         | 46             | 35 (55)         | 11 (50) | 0.81 |
| Male                           | 40             | 29 (45)         | 11 (50) |      |
| Age (y)                        | 70.1 ± 11.6    | 70.5 ± 11.8     | 68.8 ± 11.3 | 0.54 |
| Tumor site                     |                |                 |      |    |
| Colon                          | 48             | 33 (52)         | 15 (68) | 0.22 |
| Rectum                         | 38             | 31 (48)         | 7 (32)  |      |
| Degree of differentiation of tumor |        |                 |      |    |
| Good                           | 12             | 9 (14)          | 3 (15)  | 0.89 |
| Moderate                       | 61             | 46 (72)         | 15 (75) |      |
| Poor                           | 11             | 9 (14)          | 2 (10)  |      |
| Five-year overall survival     |                |                 |      |    |
| Alive                          | 33             | 20 (31)         | 13 (59) | 0.025|
| Dead                           | 53             | 44 (69)         | 9 (41)  |      |

*P calculated using Fisher’s exact test or Mann-Whitney test for the comparison of high and low SMAD4.

| Table 2. Clinical features of the Dukes C patients whose colorectal tumors were evaluated for allelic imbalance |
|---------------------------------|----------------|----------------|
|                                | Total | No 18q Al, n (%) | 18q Al, n (%) | P*   |
| Sex                            |       |                 |              |      |
| Female                         | 34    | 19 (54)         | 15 (58)      | 1.00 |
| Male                           | 27    | 16 (46)         | 11 (42)      |      |
| Age (y)                        | 70.9 ± 11.4 | 70.2 ± 12.7    | 71.9 ± 9.4 | 0.74 |
| Tumor site                     |       |                 |              |      |
| Colon                          | 34    | 22 (63)         | 12 (46)      | 0.29 |
| Rectum                         | 27    | 13 (37)         | 14 (54)      |      |
| Degree of differentiation of tumor |       |                 |              |      |
| Good                           | 9     | 3 (9)           | 6 (23)       | 0.18 |
| Moderate                       | 46    | 28 (80)         | 18 (69)      |      |
| Poor                           | 5     | 4 (11)          | 1 (4)        |      |
| Five-year overall survival     |       |                 |              |      |
| Alive                          | 24    | 13 (37)         | 11 (42)      | 0.68 |
| Dead                           | 37    | 22 (63)         | 15 (58)      |      |

NOTE: Sixty-one of the 86 patients in this study were informative for at least one of the two markers used to assess allelic imbalance (D18S1110 and D18S156).

Abbreviation: AI, allelic imbalance.

*P calculated using Fisher's exact test or Mann-Whitney test for the comparison of high and low SMAD4.
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 (26–28). The percentage of tumor cells was >50% in 84 of the 86 samples (97.6%). The specimens representing normal mucosa were always derived from a separate site rather than from the tumor margins.

Two polymorphic microsatellite markers in 18q21 within 2 Mb of SMAD4 (D18S1110 and D18S1156) were used to assess the presence of allelic imbalance in this region. The PCR primers used were D18S1110-F: 5’-TGACTTTGGCCTACCCTTG; D18S1110-R: 5’-TCCGAAAGCCT- TAAACCTGTA, D18S1156-F: 5’-CCTGAAGTTNACTGGC, and D18S1156-R: 5’-CAATGGACACCTGTGTGG. Forward primers were HEX labeled. PCR reactions were done in 15 μL reaction volume containing 50 ng genomic DNA, 1 × PCR buffer (Applied Biosystems, Foster City, CA), 130 μmol/L of each diethylthiophenyl thiophosphate (dNTP; Finnzymes, Espoo, Finland), 0.66 μmol/L each forward and reverse primer, and 0.75 units of AmpliTaqGOLD polymerase (Applied Biosystems). The following PCR cycles were used for amplification: D18S1110, 95°C for 10 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 75 seconds, and 72°C for 75 seconds; D18S1156: 95°C for 10 minutes, 30 cycles of 95°C for 30 seconds, 56°C for 75 seconds, and 72°C for 75 seconds. The size of the amplified PCR products was assessed using an Applied Biosystems ABI3730 Automatic DNA sequencer. Allelic imbalance was scored if there was a difference greater than 40% in the abundance of an allele between normal and tumor samples (29).

**Mutation analysis of SMAD4.** Sufficient DNA was available for sequencing of the full genomic coding sequence of SMAD4 (exons 1-11) for 80 of the 86 tumor samples entered into this study. DNA samples were PCR amplified using previously reported primers and conditions (30). Direct sequencing of the PCR products was done using cycle sequencing with Big Dye Terminator kit (Applied Biosystems), and reactions were run on ABI 3100 capillary sequencer (Applied Biosystems) according to the manufacturer’s instructions. The corresponding fragment from normal tissue DNA samples was ampliﬁed and sequenced for patients showing genomic alterations in the tumor samples.

**Results**

**Low SMAD4 protein levels are associated with poor prognosis in colorectal cancer.** Although DCC and SMAD2 have been suggested to be the target for most of the deletions in chromosome 18q observed in colorectal tumors (3–5), there has been increasing interest in SMAD4 as a potential target gene. SMAD4 mutations have been linked to juvenile polyposis, a colorectal cancer predisposition syndrome (9), and frequent point mutations have been observed in sporadic colorectal tumors (11, 12), suggesting that this gene is an important target for 18q deletions.

Because 18q deletions have been shown to be of prognostic significance for a subset of colorectal patients with locally advanced disease, we decided to investigate the potential of SMAD4 protein levels as a marker of prognosis in Dukes C colorectal cancer patients using immunohistochemistry. To this end, we constructed a tissue microarray containing triplicate tumor samples from all 86 Dukes C patients entered in this study. Sections of this tissue microarray were immunostained using a commercially available monoclonal antibody that has previously been shown to specifically recognize SMAD4 (24, 25).

SMAD4 protein levels were assessed independently by two researchers blinded from the clinical data using a semiquantitative scale ranging from 0 (no staining) to 4 (strong staining; Fig. 1). Excellent correlation was observed between these two independent assessments (Spearman’s R = 0.86, P < 0.0001).

To investigate the role of tumor SMAD4 levels in patient survival, we divided the 86 patients in the study into two groups depending on the intensity of SMAD4 immunostaining. Tumors with staining levels of two or less (64 of 86 tumors, 74%) were considered as low SMAD4, and staining intensities >2 (22 of 86 tumors, 26%) were considered as high SMAD4. Patients with high SMAD4 levels had significantly longer overall and disease-free survival than those with low levels of SMAD4 immunostaining (log-rank test P = 0.025 and P = 0.013, respectively; Fig. 2), indicating that low level of SMAD4 protein is a marker of poor prognosis in Dukes C colorectal cancer. No significant associations were observed between SMAD4 protein levels and patient sex, age, tumor location (colon/rectum), and grade (Table 1).

**Allelic imbalance in chromosome 18q.** Allelic losses in 18q have been shown to be a useful marker of prognosis in Dukes B colorectal cancer patients (19, 21, 31). However, there are a number of conflicting reports in the literature concerning the potential of allelic loss in 18q as a prognostic marker in Dukes C patients (18–23, 32).

This led us to investigate the prognostic significance of genetic abnormalities in chromosome 18q in this series of 86 Dukes C colorectal tumors from patients that had surgery as the only form of treatment. Two polymorphic microsatellite markers (D18S1110 and D18S1156) in 18q21 were used to investigate the presence of allelic imbalance in this region. Of the 86 tumors, 25 (29%) were either homozygous for both markers or did not amplify in the PCR reactions. Of the remaining 61 samples, 26 (42%) showed allelic imbalance in at least one of these two markers, whereas the remaining 35 (58%) showed no evidence of allelic imbalance (Table 2). In agreement with previous studies (18, 21, 23, 32), the overall survival and the disease-free survival of patients with allelic imbalance in 18q was not significantly different from that of patients without allelic imbalance (log-rank test, P = 0.79 and P = 0.37 respectively; Fig. 3).

**Mutation analysis of SMAD4.** Direct DNA sequencing of all SMAD4-coding sequences in 80 of the 86 tumor samples

![Fig. 1. SMAD4 immunohistochemical staining of colorectal tumors. Representative examples of tumors showing high (A), low (B), or absent (C) immunostaining (BB monoclonal antibody, Santa Cruz). Top, SMAD4 immunostainings; bottom, corresponding H&E-stained samples.](https://www.aacrjournals.org/clinica/canres20051107/2608/fig1.png)
entered into this study identified five-point mutations (6.25%): one truncating mutation at codon 135 and four missense mutations in codons 330, 491, 493, and 533 (Table 3).

**Correlation between SMAD4 mutations, allelic imbalance in chromosome 18q, and SMAD4 protein levels.** We next investigated if SMAD4 mutations were associated with the presence of allelic imbalance in 18q and/or reduced SMAD4 protein expression. Four of the five tumors carrying SMAD4 mutations also showed allelic imbalance in chromosome 18q21, suggesting that the wild-type allele was lost in these tumors. However, missense mutations in SMAD4 did not result in low SMAD4 protein levels (average staining level of 2.2) and the truncating mutation observed resulted in only moderately low protein levels (staining level of 1). Moreover, although two of the five patients with tumors carrying SMAD4 mutations died within 2 years of the initial surgery, another two patients had a disease-free survival that exceeded 8 years. The remaining patient died of unrelated causes 11 months after surgery.

We then examined if losses of genetic material in chromosome 18q21 had a direct effect on SMAD4 protein levels in the 61 samples that were informative for allelic imbalance. All four tumors with absent SMAD4 immunostaining had allelic imbalance in 18q21. However, patients with high or low SMAD4 levels were equally likely to have 18q21 deletions (\( P = 0.76 \), Fisher's exact tests), suggesting that other factors can modulate SMAD4 protein levels.

**Discussion**

Deletions in the long arm of chromosome 18 are among the most common genetic abnormalities found in colorectal tumors (1, 2) and the effect of this deletion in prognosis of patients with locally advanced disease has been extensively studied (19–21). An immunohistochemical assay capable of assessing the expression level of the gene or genes targeted by these frequent deletions in 18q could constitute a useful prognostic marker for Dukes C colorectal cancer patients. Several genes have been proposed as the target of these deletions including DCC and two genes involved in the signal transduction cascade initiated by TGF-β superfamily, SMAD2 and SMAD4 (3–5). TGF-β signaling is an important regulator of proliferation, differentiation, and apoptosis with a key role in nontransformed human colon epithelium homeostasis (33–35). SMAD4 has recently attracted considerable interest as a prime candidate target gene for the 18q deletions because of recent data linking mutations in this gene to sporadic and familial colorectal cancer (9, 11, 12).

In this study, we investigated the potential of SMAD4 protein levels as a prognostic factor for Dukes C colorectal cancer patients using an immunohistochemical approach. The median overall survival of patients with low SMAD4 levels was 1.7 years, whereas for patients with high SMAD4 levels it was >9 years.
SMAD4 studies linking increased Dukes C colorectal cancer. This is in agreement with previous protein level is a marker of poor prognosis for patients with \( P = 0.013 \), respectively), indicating that low SMAD4 tumor Kaplan-Meier analysis of the survival curves showed a significantly worse overall and disease-free survival for patients whose tumors had low SMAD4 levels (log-rank test, \( P = 0.025 \) and \( P = 0.013 \), respectively), indicating that low SMAD4 tumor protein level is a marker of poor prognosis for patients with Dukes C colorectal cancer. This is in agreement with previous studies linking increased SMAD4 mutation frequency and reduced SMAD4 mRNA levels with tumor progression (12, 14).

Approximately 50% of Dukes C patients show disease recurrence and die of their disease within 5 years of surgery, and it is currently not possible to accurately distinguish surgically cured patients from those at high risk of recurrence (36, 37). SMAD4 levels could constitute a useful prognostic marker for these patients, identifying patients that are more likely to have disease recurrence and are, thus, good candidates to receive an aggressive adjuvant chemotherapeutic treatment. 5-Fluorouracil–based adjuvant treatment has been shown to prevent disease recurrence in 10% to 20% of Dukes C patients, and has now become standard treatment for Dukes C patients (36–38). However, additional chemotherapeutic agents, such as CPT-11 and oxaliplatin, are now available for the treatment of these patients and could be used in combination with 5-fluorouracil in an attempt to improve the long-term survival of these patients. Importantly, the tumor samples used in this study were collected before adjuvant chemotherapy became routine treatment for these patients in the collaborating institutions, and, therefore, the interpretation of these results is not complicated by the possible role of SMAD4 in patient response to chemotherapy.

To investigate the possible impact of SMAD4 mutations in patient survival and protein expression levels, we sequenced all the SMAD4 coding sequences (exons 1-11). In agreement with the low number of mutations previously reported in locally advanced sporadic colorectal tumors (12), we found five somatic mutations in the 80 tumors with enough DNA available for this analysis (6.25%). In four of these five tumors, there was evidence of allelic imbalance in 18q, the chromosomal location of SMAD4, suggesting the loss of the wild-type allele in these tumors and a lack of SMAD4 function. Although two of the five patients with tumors carrying SMAD4 mutations had disease recurrence within 2 years of surgery, the low incidence of mutations in these patients limits the value of SMAD4 mutations as a prognostic marker.

We next investigated the effects of the observed 18q deletions on SMAD4 protein levels. If SMAD4 is the target of these deletions, the presence of 18q allelic imbalance could be expected to be associated with reduced SMAD4 protein levels. Of the 61 informative samples in this study, 26 (42%) showed allelic imbalance in 18q21. Among these 61 samples, four displayed a total lack of SMAD4 immunostaining and these four tumors also showed allelic imbalance in 18q21. However, when all 61 tumors with data available for both allelic imbalance and SMAD4 levels are considered, there was no correlation between allelic imbalance and SMAD4 levels. The lack of a perfect correlation between deletions in 18q and reduced SMAD4 levels has been reported before in colorectal and ovarian tumors (24, 39) and indicates that heterozygous deletion of this gene does not necessarily lead to a reduction in SMAD4 protein levels. Moreover, additional mechanisms must exist to down-regulate SMAD4 in tumor cells. Although SMAD4 point mutations are found in ~50% of pancreatic carcinomas (40), inactivating mutations of this gene are less frequent in colorectal tumors (11, 12). Whereas over 40% of Dukes C colorectal tumors have deletions in 18q, previous studies have identified SMAD4 mutations in <10% of these tumors (11, 12). Here, we show that mutations in SMAD4 can result in reduced SMAD4 protein levels, but the low number of mutations found cannot account for the large proportion of tumors exhibiting low SMAD4. Hypermethylation of CpG islands within the promoter sequences in several tumor suppressor genes, such as MLH1 (41) and p16 (42), has been linked to reduced gene expression, but promoter hypermethylation of SMAD4 has not been observed in intestinal tumors (43). As suggested before (44), the lack of correlation observed between allelic imbalance in 18q and low SMAD4 levels also suggests that more than one tumor suppressor gene may be targeted by these deletions in chromosome 18q.

A number of studies have shown that allelic loss in 18q is associated with poor prognosis in Dukes B colorectal tumors (19, 20). However, the significance of these deletions in the survival of lymph node–positive Dukes C patients is still controversial, with some studies showing improved survival for patients retaining heterozygosity in this region (19, 20, 22) and other studies showing no prognostic value (18, 21, 24, 32). In this study, we found no significant difference in overall or disease-free survival of patients with or without allelic imbalance in this region (log-rank test, \( P = 0.79 \) and \( P = 0.37 \), respectively).

Collectively, these results suggest that immunohistochemical analysis of SMAD4 protein level is a more sensitive prognostic marker for Dukes C patients than SMAD4 mutations or 18q allelic imbalance and could be used to identify a subset of patients with elevated risk of recurrence.

Table 3. Mutations detected in the 80 colorectal tumors sequenced

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino acid change</th>
<th>18q AI</th>
<th>SMAD4 immunohistochemistry</th>
</tr>
</thead>
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<tr>
<td>c438</td>
<td>2</td>
<td>135</td>
<td>CGA → TGA</td>
<td>Arg → Stop</td>
<td>Al</td>
<td>1</td>
</tr>
<tr>
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<td>330</td>
<td>GAA → AAA</td>
<td>Glu → Lys</td>
<td>Al</td>
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<tr>
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<td>GGT → GTT</td>
<td>Gly → Val</td>
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</tr>
<tr>
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<td>Asp → Val</td>
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<td>Leu → Val</td>
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</table>

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SMAD4 and Prognosis in Colorectal Cancer

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