Acceleration of Smad2 and Smad3 Phosphorylation via c-Jun NH2-Terminal Kinase during Human Colorectal Carcinogenesis

Hideo Yamagata, Koichi Matsuzaaki, Shigeo Mori, Katsunori Yoshida, Yoshiya Tahashi, Fukiko Furukawa, Go Sekimoto, Toshihiko Watanabe, Yoshiro Uemura, Noriko Sakaida, Kazuhiko Yoshioka, Yasuo Kamiyama, Toshihito Seki, and Kazuichi Okazaki

Abstract
Conversion of normal epithelial cells to tumors is associated with a shift in transforming growth factor-β (TGF-β) function: reduction of tumor suppressor activity and increase of oncogenic activity. However, specific mechanisms of this functional alteration during human colorectal carcinogenesis remain to be elucidated. TGF-β signaling involves Smad2/3 phosphorylated at linker regions (pSmad2/3L) and COOH-terminal regions (pSmad2/3C). Using antibodies specific to each phosphorylation site, we herein showed that Smad2 and Smad3 were phosphorylated at COOH-terminal regions but not at linker regions in normal colorectal epithelial cells and that pSmad2/3C were located predominantly in their nuclei. However, the linker regions of Smad2 and Smad3 were phosphorylated in 31 sporadic colorectal adenocarcinomas. In particular, late-stage invasive and metastatic cancers typically showed a high degree of phosphorylation of Smad2/3L. Their extent of phosphorylation in 11 adenomas was intermediate between those in normal epithelial cells and adenocarcinomas. Whereas pSmad2L remained in the cytoplasm, pSmad3L was located exclusively in the nuclei of Ki-67-immunoreactive adenocarcinomas. In contrast, pSmad3C gradually decreased as the tumor stage progressed. Activated c-Jun NH2-terminal kinase in cancers could directly phosphorylate Smad2/3L. Although Mad homology 2 region sequencing in the Smad4 gene revealed a G/A substitution at codon 361 in one adenocarcinoma, the mutation did not correlate with phosphorylation. No mutations in the type II TGF-β receptor and Smad2 genes were observed in the tumors. In conclusion, pSmad3C, which favors tumor suppressor activity of TGF-β, was found to decrease, whereas c-Jun NH2-terminal kinase tended to induce the phosphorylation of Smad2/3L in human colorectal adenoma-carcinoma sequence. (Cancer Res 2005; 65(1): 157-65)

Introduction
The concept of adenoma-carcinoma sequence is widely accepted concerning sporadic colorectal carcinogenesis (1). On the other hand, emerging evidence indicates that transforming growth factor-β (TGF-β) signaling participates not only in tumor suppressor activities such as growth inhibition and apoptosis but also in oncogenic processes such as growth stimulation, increases in motility, invasion, and metastasis (2). Conversion of non-tumorigenic phenotype of human colonic adenoma cell lines to a tumorigenic phenotype is associated with reduced tumor suppressor activities and increased oncogenic activities of TGF-β (3). Modulation of growth factor effects can be achieved by various mechanisms, including changes in ligand concentration, activation of latent forms of the ligand, modulation of number and affinity of receptors, and alterations in postreceptor pathways.

Progress over the past several years has disclosed some details of how TGF-β elicits its responses. TGF-β signaling is initiated when this ligand induces formation of a heteromeric complex composed of TGF-β receptor type I (TβRI) and type II (TβRII; ref. 4). This allows TβRII to phosphorylate TβRI, which then transmits the signal through phosphorylation of receptor-regulated Smads such as Smad2 and Smad3 (5). Both of those Smads are directly phosphorylated at COOH-terminal SXS regions by TβRI and then undergo formation of heteromeric complexes with Smad4 (6). Activated Smad complexes then are translocated into the nucleus, where they regulate expression of target genes both by direct DNA binding and through interaction with other transcription factors, coactivators, and corepressors (7). Smads contain two highly conserved domains, the Mad homology 1 (MH1) and 2 (MH2) domains, which are connected by interposed linker regions (8). Although their MH1 domains can interact with DNA, the MH2 domains are endowed with transcriptional activation properties (6).

The TβRII/Smad pathway is widely represented in most cell types and tissues studied to date, and additional pathways are activated following cell stimulation by TGF-β in specific contexts. The most prominent pathways are mediated by the mitogen-activated protein kinase (MAPK) family, which consists of the extracellular signal-regulated protein kinase pathway and two stress-activated protein kinase pathways: c-Jun NH2-terminal kinase (JNK) and p38 pathways (9). TGF-β induces activation of MAPK pathways through the upstream mediators Ras, RhoA, PP2A, and TGF-β-activated kinase 1 (10). To investigate the roles of Smad2 and Smad3 phosphorylation in TGF-β signal transduction, we developed four types of polyclonal antibodies (Abs) in our laboratory that specifically recognized the phosphorylated linker regions and the phosphorylated COOH-terminal SXS regions in Smad2 and Smad3 (11). Studies using the Abs showed that TGF-β signal phosphorylated Smad2 and Smad3 not only at COOH-terminal SXS regions but also at linker regions. Smad2 or Smad3 phosphorylated at linker regions or COOH-terminal regions existed in motility, invasion, and metastasis (2). Conversion of non-tumorigenic phenotype of human colonic adenoma cell lines to a tumorigenic phenotype is associated with reduced tumor suppressor activities and increased oncogenic activities of TGF-β (3).
as separate molecules with different functions and transmitted distinct signals. In particular, JNK and/or p38 MAPK activated on TGF-β treatment could directly phosphorylate Smad2 and Smad3 at linker regions. Moreover, TGF-β treatment led to an increase in plasminogen activator inhibitor type 1 transcriptional activity through pSmad3L (11).

Whereas JNK signals can modify TβRII-mediated signaling in vitro, proof has been lacking that this event occurs in vivo. Additionally, the cellular distribution of phosphorylated Smad2 and Smad3 has not been studied in human tissues. Without Abs to selectively distinguish phosphorylation sites in Smad2 and Smad3, determination of phosphorylation sites and investigation of their distinct phosphorylated domain-mediated signals in vivo has been difficult. Using domain-specific phospho-Smad2/3 Abs, we carried out the present study to elucidate how pSmad2/3-mediated signals changed during human colorectal carcinogenesis. Our results indicated that JNK activation occurred during progression to malignancy, accompanied by apparent Smad2/3 phosphorylation at linker regions in situ. Moreover, JNK in cancerous tissues could directly phosphorylate Smad2 and Smad3 at linker regions. In particular, Smad3 phosphorylated at the linker region was localized predominantly to cell nuclei in actively growing Ki-67-immunoreactive adenocarcinoma with distant metastasis. Collectively, Smad2 and Smad3 phosphorylation at linker regions could play an important role in transmitting JNK-mediated signals in human sporadic colorectal cancer.

Materials and Methods

Tumor Specimens. According to criteria of the Japanese Classification of Colorectal Carcinoma (12), the principal histologic distinction is between benign epithelial tumors including adenoma and malignant epithelial tumors including adenocarcinoma. Pathology records and histologic slides involving diagnoses of either adenoma or adenocarcinoma made from 2000 to 2003 in the Department of Surgical Pathology at Kansai Medical University Hospital were reviewed independently by two pathologists with an interest in gastrointestinal neoplasia (Y.U. and N.S.).

This study was approved by the Ethics Committee of Kansai Medical University. Seventeen men and 14 women, ages 47 to 88 years at diagnosis, with their primary colorectal tumor (n = 35) and a peritumoral specimen (n = 35) were enrolled in this study. The 2000 to 2003 Cancer Registry database at Kansai Medical University provided the following clinicopathologic parameters: age, sex, site, stage, histologic type, and survival for 152 colorectal cancer patients. Their clinicopathologic features are shown in Table 1. Tumor specimens were fixed in 3% formalin for 2 to 3 days, dehydrated through graded alcohol series, embedded in paraffin, and sectioned at a thickness of 4 μm. Paraffin sections then were deparaffinized in xylene and rehydrated. Nonenzymatic antigen retrieval was done by heating sections to 121 °C in 0.01 mol/L sodium citrate buffer (pH 6.0) for 10 minutes. After cooling, sections were rinsed in TBS containing 0.1% Tween 20 (TBST) and incubated in methanol-3% H2O2 for 30 minutes to quench endogenous peroxidase activity. After rinsing with TBST, sections were incubated with primary Abs for 1 hour at room temperature in a humid chamber. Primary Abs used in this study included mouse monoclonal Ab anti-Ki-67 (0.8 μg/mL, DAKO, Glostrup, Denmark), anti-pSmad2L (0.5 μg/mL), anti-pSmad2C (0.5 μg/mL), anti-pSmad3L (1 μg/mL), and anti-pSmad3C (1 μg/mL). Anti-pSmad3C Ab cross-reacted weakly with COOH-terminally phosphorylated Smad2. To block binding of anti-pSmad3C Ab to phosphorylated domains in Smad2, anti-pSmad3C Ab was adsorbed with 1 μg/mL COOH-terminally phosphorylated Smad2 peptide. After sections were rinsed in TBST, they were incubated with peroxidase-labeled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulin (DAKO) for 1 hour at room temperature. Finally, the sections were developed with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA), counterstained with Mayer’s hematoxylin (Merk, Darmstadt, Germany), and mounted under coverslips.

Immunoprecipitation and Immunoblotting. Frozen tissues were extracted with TNE buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 100 mmol/L phenylmethylsulfonyl fluoride]. Cell extracts were subjected to immunoprecipitation with monoclonal anti-Smad2/3 Ab (BD Transduction Laboratories, Lexington, KY) followed by adsorption to protein G-Sepharose (Pharmacia, Peapack, NJ) for 1 hour. After washing thrice with TNE buffer, the immunoprecipitates were separated by 7.5% SDS-PAGE, and transferred to a nitrocellulose membrane (Pharmacia). After the membranes were blocked overnight in TBST containing 5% bovine serum albumin (Nakarai, Kyoto, Japan), they were incubated with the indicated primary Abs for 1 hour at room temperature. Extent of phosphorylation of Smad2 and Smad3 was determined using each anti-pSmad2/3 Ab (11). Immunoblotting of total cell extracts was carried out using rabbit polyclonal anti-pSmad2/3 Ab (Cell Signaling Technology, Beverly, MA), anti-p38 MAPK Ab (Promega, Madison, WI), anti-JNK1/2 Ab (Cell Signaling Technology), and anti-p38 MAPK Ab (Cell Signaling Technology). After the membranes were rinsed in TBST, they were incubated with horseradish peroxidase–conjugated anti-rabbit polyclonal Ab for 1 hour at room temperature. Proteins were detected by enhanced chemiluminescence (Pharmacia) and autoradiography. Densities of immunoreactive bands were measured using a densitometer (LKB, Bromma, Sweden).

In vitro Kinase Assay. Bacterial expression and purification of GST-Smad2 and GST-Smad3 were carried out according to the manufacturer's instructions (Amersham Biosciences, Piscatway, NJ). Endogenous kinases were isolated from the protein extracts using anti-pSmad1/2 Ab (Promega). Immune complexes collected with protein G-Sepharose were washed with kinase assay buffer [25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na3VO4, 10 mmol/L MgCl2]. Pellets were resuspended in kinase assay buffer supplemented with 100 mmol/L ATP and 2 μg of bacterially expressed GST-Smad2 or GST-Smad3. Assays were carried out at 30°C for 30 minutes and then were stopped by addition of Laemmli sample buffer. Phosphorylation sites in

Cancer Research 2005; 65: (1). January 1, 2005
Smad2 and Smad3 were determined by immunoblotting using each anti-
pSmad2/3 Ab.

**Statistical Analysis.** Statistical evaluation was done using the nonpa-
rametric Wilcoxon and Mann-Whitney _U_ ranking tests. All values were based
on two-tailed statistical analysis. The first test was used to evaluate significant
differences in Smad2/3 phosphorylation between 31 paired primary adeno-
carcinomas and uninvolved normal colorectal mucosa from the same patients. The Mann-Whitney _U_ test was used to test significant
differences in Smad2/3 phosphorylation between 11 adenomas and
uninvolved normal colorectal mucosa in the patients with colorectal
cancer. _P_ < 0.05 was considered significant.

**Results**

**DNA Sequencing of RT-PCR Products from 31 Human Colorectal Adenocarcinomas and 11 Adenomas Detects One
Mutation of the Smad4 Gene in One Case.** Mutations of _TβRII_, Smad2, and Smad4 genes have been detected in human colorec-
tal tumors (14–17). To clarify the contribution of TGF-β signaling in colorectal carcinogenesis, we initially analyzed mutations of
these genes in 31 adenocarcinoma and 11 adenoma samples. The _TβRII_ gene was amplified by RT-PCR using primers that included extracellular and kinase domains (18). RT-PCR primers for Smad2 and Smad4 genes included the MH2 domains, which are involved in homo- and hetero-oligomerization (6, 19, 20). When the PCR products from the tumor samples were sequenced, we detected a G/A substitution in one sample at codon 361, resulting in a missense mutation (Arg-to-His; patient 15 in Table 1). We detected no mutations in _TβRII_ or Smad2 genes from any colorectal tumor samples. This represented a rela-
tively low mutation frequency, which is consistent with recent reports (21).

**Smad2/3 Phosphorylation at Linker Regions Is Accelerated
during Late Progression of Colorectal Tumors.** TGF-β signaling
involves phosphorylation of Smad2 and Smad3 (5). Both Smad2
and Smad3 possess two major phosphorylation sites: the linker
region and the COOH-terminal region. The former and the latter are phosphorylated via the MAPK pathway and TβRIL,
respectively (6). To investigate pSmad2/3-mediated signaling _in vivo_, we produced four Abs specific to each phosphorylated site (Fig. 1A).4 Studies using the Abs revealed that Smad3 phos-
phorylated at the linker region and that phosphorylated at the COOH-terminal region existed as separate molecules and trans-
mitted distinct signals.5 Accordingly, each molecule, including Smad2/3 phosphorylated at COOH-terminal regions (pSmad2C
and pSmad3C) and Smad2/3 phosphorylated at linker regions (pSmad2L and pSmad3L), can be localized in different cell types and
can be detected in different intracellular locations even in the same
cell.

Figure 1B and C show the distribution of phosphorylated Smad2/3 molecules in human colonic adenocarcinoma and
uninvolved normal colonic mucosa from patient 6 in Table 1. Immunostaining with Abs against Smad2/3 at COOH-terminal
regions indicated that Smad2/3 in normal colonic epithelial cells were phosphorylated at COOH-terminal regions and that pSmad2C
and pSmad3C were located predominantly in nuclei (Fig. 1B and
C, right, _α_ pSmad2C and _α_ pSmad3C). The phosphorylation level
of Smad2C in adenocarcinoma was almost the same as that in
normal epithelial cells, and pSmad2C was distributed evenly in the
nuclei of the adenocarcinoma cells (Fig. 1B and C, left compared with
right, _α_ pSmad2C). However, nuclear pSmad3C immunostaining
showed a scattered distribution throughout the adenocarcinoma
specimen (Fig. 1B and C, left, _α_ pSmad3C). In contrast to highly phos-
phorylated states of pSmad2C and pSmad3C, pSmad2L and pSmad3L showed little phosphorylation in normal colonic epithelial cells
(Fig. 1B and C, right, _α_ pSmad2L and _α_ pSmad3L). However, both
Smad2 and Smad3 were highly phosphorylated at linker regions in
adenocarcinomas (Fig. 1B and C, left, _α_ pSmad2L and _α_ pSmad3L). Subcellular localization of pSmad3L was different from that of pSmad2L. Whereas pSmad2L remained in the cytoplasm of the ad-
enoarcinoma, pSmad3L accumulated in tumor cell nuclei (Fig. 1C, left, _α_ pSmad2L and _α_ pSmad3L).

Figure 1D illustrates the distribution of phosphorylated Smad2/3 molecules in a human colonic adenoma. As in normal epithelial
cells, both Smad2 and Smad3 were phosphorylated at COOH-
terminal regions and pSmad2C and pSmad3C were located predominantly in the nuclei of adenoma cells (Fig. 1D, _α_ pSmad2C
and _α_ pSmad3C). Similarly, Smad2 and Smad3 were phos-
phorylated at linker regions in the adenoma (Fig. 1D, _α_ pSmad2L
and _α_ pSmad3L). Their extent of phosphorylation in the adenoma
was intermediate between those in normal epithelial cells and
adenocarcinomas. Whereas pSmad2L was found mainly in the
cytoplasm of the adenoma cells, pSmad3L accumulated in the
nuclei of these cells.

To evaluate extent of phosphorylation of each domain in Smad2
and Smad3, we subjected the extracts from colorectal tumors and
uninvolved normal mucosa to immunoblotting with domain-
specific Abs against the phosphorylated Smads. The linker regions
of Smad2 and Smad3 showed very little phosphorylation in normal
mucosa, where the COOH-terminal regions of Smad2 and Smad3
were moderately phosphorylated (Fig. 2A). In contrast, Smad2 and
Smad3 in adenocarcinomas were highly phosphorylated at linker
regions. Although the extent of phosphorylation of Smad2C in
adenocarcinomas was almost the same as that in normal mucosa,
Smad3C showed somewhat less phosphorylation in primary
invasive adenocarcinoma than in normal mucosa. Considering
immunoblotting findings together with the results obtained from
immunohistochemical analyses (Fig. 1B and C), Smad2 and Smad3
were constitutively phosphorylated at linker regions in adenocar-
cinomas.

We then densitometrically quantified Smad2/3 phosphorylation
in tumor tissues representing various tumor stages. In the
adenoma, pSmad2L was increased a few times beyond the amount
present in normal mucosa (Fig. 2B). A correlation was observed between pSmad2L and stage of colorectal cancer. Thus, pSmad2L
in primary invasive adenocarcinoma (stages III and IV) averaged
five times the amount in uninvolved mucosa. Phosphorylation of
Smad2C showed slight up-regulation in adenoma and cancer
samples (Fig. 2C). Remarkable up-regulation of pSmad3L was seen
in the process of human colorectal carcinogenesis (Fig. 2D). In
particular, late-stage invasive and metastatic cancers typically
showed a high degree of phosphorylation of Smad3L. In contrast,
pSmad3C gradually decreased as the tumor stage progressed
(Fig. 2E; ref. 11).

**JNK in Human Colorectal Adenocarcinoma Directly Phos-
phorylates Smad2/3 at Linker Regions.** We reported previously
that Ser39/254 in Smad2 and Ser387/212 in Smad3, which anti-
pSmad2L Ab and anti-pSmad3L Ab recognized, respectively,
served as substrates for JNK and/or p38 MAPK _in vitro_ after
TGF-β treatment (11).6 Accordingly, we further investigated the
phosphorylation states of JNK1/2 and p38 MAPK in the process
of human colorectal carcinogenesis. We subjected the ex-
tracts from colorectal tumors and uninvolved normal mucosa to

---

4. The Abs used in this study were raised against recombinant Smad2 and Smad3 proteins and were shown to be specific and reliable by Western blotting and immunohistochemical analysis.

5. The results indicated that Smad2 and Smad3 signaling pathways are activated at different stages of colorectal tumor development.

6. Serine/threonine-specific kinases, such as JNK and p38 MAPK, have been implicated in the regulation of Smad phosphorylation in response to TGF-β signaling.
immunoblotting with Abs specific for phosphorylated JNK1/2 and p38 MAPK. The phosphorylation of JNK1, but not JNK2, was increased 2.4-fold in late-stage invasive and metastatic cancers compared with normal mucosa (Fig. 3A and B). In contrast, the degree of p38 MAPK phosphorylation in cancerous tissues was almost the same as that in uninvolved mucosa (Fig. 3A and C).

Because the profiles of Smad2 and Smad3 phosphorylation in specimens representing stages in human colorectal carcinogenesis resembled those of JNK1 activation, these events may be causally linked.

To address the functional relationship between activated JNK and Smad2/3 phosphorylation in human colorectal tumors, we next assayed kinase activity in vitro. In proportion to the low activities of JNK1/2 in normal mucosa, JNK showed little ability to phosphorylate Smad2 or Smad3 at linker regions (Fig. 4A). In contrast, JNK from human colorectal adenocarcinoma could directly phosphorylate Smad2 and Smad3 at linker regions. Taken together with the nuclear localization of pSmad3L in human colorectal adenocarcinoma (Fig. 1B and C), the results showed that JNK directly phosphorylated the linker region of Smad3 leading to translocation of the protein into the nuclei of the adenocarcinoma (11). Examined as a control, activated JNK failed to phosphorylate Smad2 and Smad3 at COOH-terminal regions (data not shown).

Quantification of pSmad2/3L signals in tumor tissues revealed that JNK-mediated pSmad2/3L was up-regulated during the process of human colorectal carcinogenesis (Fig. 4B and C). In particular, late-stage invasive and/or metastatic cancers showed high activities of JNK, phosphorylating Smad2/3L.

Growing Ki-67-Immunoreactive Adenocarcinoma with Distant Metastasis Shows pSmad3L Localization Exclusively in Cell Nuclei. We finally investigated the relationship between pSmad3L and cellular proliferation in primary invasive adenocarcinoma with distant metastasis. To assess the proliferative states of adenocarcinoma, nuclear expression of proliferation-associated antigen Ki-67 was examined immunohistochemically.

### Table 1. Clinicopathologic features, Smad2/3 phosphorylation, and Smad4 mutation in specimens from patients with colorectal adenocarcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumor</th>
<th>Stage (pTNM)</th>
<th>Differentiation</th>
<th>Phosphorylation*</th>
<th>Mutation Smad4 (codon361)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pS2L</td>
<td>pS2C</td>
</tr>
<tr>
<td>1</td>
<td>I</td>
<td>T1N0M0</td>
<td>Well</td>
<td>2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>2.1</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>3.3</td>
<td>1.3</td>
</tr>
<tr>
<td>4</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>4.2</td>
<td>1.3</td>
</tr>
<tr>
<td>5</td>
<td>II</td>
<td>T3N0M0</td>
<td>Moderate</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>3.5</td>
<td>1.4</td>
</tr>
<tr>
<td>7</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>4.1</td>
<td>1.2</td>
</tr>
<tr>
<td>8</td>
<td>II</td>
<td>T3N0M0</td>
<td>Moderate</td>
<td>3.2</td>
<td>1.1</td>
</tr>
<tr>
<td>9</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>4.4</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>II</td>
<td>T3N0M0</td>
<td>Moderate</td>
<td>4.3</td>
<td>1.4</td>
</tr>
<tr>
<td>11</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>4.0</td>
<td>1.6</td>
</tr>
<tr>
<td>12</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>5.1</td>
<td>1.3</td>
</tr>
<tr>
<td>13</td>
<td>II</td>
<td>T3N0M0</td>
<td>Moderate</td>
<td>4.0</td>
<td>1.3</td>
</tr>
<tr>
<td>14</td>
<td>II</td>
<td>T3N0M0</td>
<td>Well</td>
<td>4.7</td>
<td>1.5</td>
</tr>
<tr>
<td>15</td>
<td>III</td>
<td>T3N1M0</td>
<td>Moderate</td>
<td>4.1</td>
<td>1.3</td>
</tr>
<tr>
<td>16</td>
<td>III</td>
<td>T3N1M0</td>
<td>Well</td>
<td>4.4</td>
<td>1.3</td>
</tr>
<tr>
<td>17</td>
<td>III</td>
<td>T3N2M0</td>
<td>Mucinous</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>18</td>
<td>III</td>
<td>T3N2M0</td>
<td>Well</td>
<td>4.9</td>
<td>1.6</td>
</tr>
<tr>
<td>19</td>
<td>III</td>
<td>T2N2M0</td>
<td>Moderate</td>
<td>4.7</td>
<td>1.5</td>
</tr>
<tr>
<td>20</td>
<td>III</td>
<td>T2N1M0</td>
<td>Well</td>
<td>4.1</td>
<td>1.2</td>
</tr>
<tr>
<td>21</td>
<td>III</td>
<td>T3N1M0</td>
<td>Well</td>
<td>5.8</td>
<td>1.3</td>
</tr>
<tr>
<td>22</td>
<td>III</td>
<td>T3N1M0</td>
<td>Moderate</td>
<td>6.4</td>
<td>1.8</td>
</tr>
<tr>
<td>23</td>
<td>III</td>
<td>T3N1M0</td>
<td>Well</td>
<td>7.5</td>
<td>1.2</td>
</tr>
<tr>
<td>24</td>
<td>IV</td>
<td>T3N0M1</td>
<td>Well</td>
<td>3.1</td>
<td>1.2</td>
</tr>
<tr>
<td>25</td>
<td>IV</td>
<td>T3N2M1</td>
<td>Well</td>
<td>3.3</td>
<td>1.5</td>
</tr>
<tr>
<td>26</td>
<td>IV</td>
<td>T3N2M1</td>
<td>Moderate</td>
<td>5.8</td>
<td>1.2</td>
</tr>
<tr>
<td>27</td>
<td>IV</td>
<td>T3N2M1</td>
<td>Well</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>28</td>
<td>IV</td>
<td>T3N3M1</td>
<td>Moderate</td>
<td>4.6</td>
<td>1.5</td>
</tr>
<tr>
<td>29</td>
<td>IV</td>
<td>T3N1M1</td>
<td>Well</td>
<td>8.7</td>
<td>1.1</td>
</tr>
<tr>
<td>30</td>
<td>IV</td>
<td>T3N3M1</td>
<td>Moderate</td>
<td>6.9</td>
<td>1.7</td>
</tr>
<tr>
<td>31</td>
<td>IV</td>
<td>T3N0M1</td>
<td>Well</td>
<td>8.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Relative to adjacent normal mucosa as determined by immunoblotting.
Figure 5 shows pSmad3L distribution and Ki-67 expression in primary invasive adenocarcinoma and uninvolved colonic mucosa from patient 29 in Table 1. Smad3L was only minimally phosphorylated in normal colonic epithelial cells (Fig. 5A); scattered pSmad3L-immunoreactive cells were essentially confined to the proliferative compartments in the basal third of the mucosal crypts (Fig. 5B). Throughout the glands formed by the adenocarcinoma, pSmad3L was significantly more abundant than in normal glands. Smad3L occasionally showed phosphorylation in inflammatory cells within mucosal connective tissue. Glands showing highly phosphorylated Smad3L were evenly distributed in the adenocarcinoma. Expression of Ki-67 was low in the normal mucosa. In particular, the superficial epithelium failed to show any Ki-67 expression. In parallel with the distribution of pSmad3L-immunoreactive cells, scattered Ki-67-immunoreactive cells were confined to the basal third of crypts. Colonic adenocarcinoma
Figure 2. Phosphorylation of Smad2/3 is up-regulated at linker regions in human colorectal adenocarcinoma. A, cell lysates obtained from colorectal cancerous tissues and uninvolved mucosa from patients 6, 19, and 29 in Table 1 and adenoma from patient 1 were subjected to anti-Smad2/3 immunoprecipitation (IP) and were immunoblotted with each anti-pSmad2/3 Ab (top). Relative amounts of endogenous Smad2/3 were determined by immunoblotting (IB) using anti-Smad2/3 Ab (bottom). Graphic analyses of immunoblots show the ratio of pSmad2L to Smad2 (B), pSmad2C to Smad2 (C), pSmad3L to Smad3 (D), or pSmad3C to Smad3 (E) in human colorectal tumor tissues. Intensities of pSmad2L, pSmad2C, pSmad3L, or pSmad3C bands were normalized to those of Smad2 or Smad3 in corresponding groups. The ratio of the phosphorylated Smad2/3 to Smad2/3 in uninvolved mucosa was assigned a value of 1. Points, mean for each group. *, P < 0.05; **, P < 0.01; NS, not significant.
Figure 3. JNK1, but not p38 MAPK, is phosphorylated in human colorectal adenocarcinoma. A, cell lysates obtained from colorectal cancerous tissues and uninvolved mucosa from patients 6, 19, and 29 in Table 1 and adenoma from patient 1 were immunoblotted with anti-phospho-JNK1/2 (α pJNK1/2) or anti-phospho-p38 MAPK (α pp38) Ab. Relative amounts of endogenous JNK1/2 and p38 MAPK were determined by immunoblotting using anti-JNK1/2 (α JNK1/2) or anti-p38 MAPK (α p38) Ab. Graphic analyses of immunoblots show the ratio of pJNK1/2 to JNK1/2 (B) or pp38 MAPK to p38 MAPK (C) in human colorectal tumor tissues. Intensities of pJNK1/2 or pp38 MAPK bands were normalized to those of JNK1/2 or p38 MAPK in corresponding groups. The ratio of the phosphorylated stress-activated protein kinase to stress-activated protein kinase in uninvolved mucosa was assigned a value of 1. Points, mean for each group. *, P < 0.05; **, P < 0.01; NS, not significant.

Figure 4. JNK in human colorectal adenocarcinoma directly phosphorylates Smad2/3 at linker regions. A, cell lysates obtained from colorectal cancerous tissues and uninvolved mucosa from patients 6, 19, and 29 in Table 1 and adenoma from patient 1 were subjected to anti-phospho-JNK1/2 immunoprecipitation and were mixed with bacterially expressed GST-Smad2 and GST-Smad3. Phosphorylation of Smad2/3L was analyzed by immunoblotting using each anti-pSmad2/3L Ab (top). Total Smad2 and Smad3 were determined by immunoblotting using anti-Smad2/3 Ab (bottom). Graphic analyses of immunoblots show the ratio of pSmad2L to Smad2 (B) or pSmad3L to Smad3 (C) in human colorectal tumor tissues. Intensities of pSmad2L or pSmad3L bands were normalized to those of Smad2 or Smad3 in corresponding groups. The ratio of the phosphorylated Smad2/3L to Smad2/3 in uninvolved mucosa was assigned a value of 1. Points, mean for each group. **, P < 0.01; NS, not significant.
showed a higher frequency of expression of Ki-67, but this still was patchy. A significant positive relationship was evident between pSmad3L distribution and Ki-67 expression, although the constitutive distribution of pSmad3L was greater than that of Ki-67.

Discussion

Conversion of normal epithelial cells to tumors is associated with a shift in TGF-β function, specifically reduction of tumor suppressor activity and increase of oncogenic activity (2). However, specific mechanisms of this functional alteration in human tumor cells and also which specific steps in carcinogenesis manifest oncogenic activities of TGF-β remain to be elucidated.

TGF-β inhibited growth of a rat intestinal epithelial cell line (22) and was found to directly induce apoptotic cell death (23). In an investigation of ligand-receptor interactions in normal colorectal epithelial cells, we reported previously that both TβRI and TβRII were strongly expressed in human normal colorectal epithelial cells (24). In situ hybridization and immunohistochemistry to detect the ligand suggested that TGF-β is produced by and is present within human colorectal epithelial cells (24, 25). Taken together with our current findings that both pSmad2C and pSmad3C were located predominantly in the nuclei of normal colorectal epithelial cells, TGF-β in normal colorectal epithelial cells mainly transmits autocrine signals through pSmad2/3C mediated by TβRI. In most normal epithelial cell types, TGF-β arrests cell cycle progression in the G1 phase by up-regulating expression of cyclin-dependent kinase inhibitors p21WAF1/CIP1 and/or p15INK4B (26, 27). TGF-β also induces expression of death-associated protein kinase as an immediately-early response by the cells in which it induces apoptosis (28).

The death-associated protein kinase promoter is activated by TGF-β through the action of Smad2, Smad3, and Smad4. Induction of both cyclin-dependent kinase inhibitors and death-associated protein kinase expression requires COOH-terminal phosphorylation of Smad2 and Smad3 (28–30). Thus, pSmad2/3C-mediated signaling seems to take part in growth inhibition and apoptosis in normal colorectal epithelial cells.

Our current findings highlighted a significant increase in JNK-dependent pSmad2/3L as the process of neoplasia progressed from normal colorectal epithelial cells to invasive adenocarcinoma with distant metastasis. In addition, nuclear pSmad3L showed a positive correlation with Ki-67 expression, a proliferative marker. These results confirm and extend previous findings that JNK activation is involved in proliferation of rat intestinal neoplasia (31). Because JNK-dependent Smad3L phosphorylation promotes nuclear accumulation of Smad3, reduction of pSmad3C could be explained by reduced accessibility of Smad3 to TβRI (11). Accordingly, an increase in pSmad3L during colorectal carcinogenesis (Fig. 2D) could lead to the observed decrease in pSmad3C (Fig. 2E), resulting in desensitization of the cell to TGF-β tumor suppressor activity (11). Moreover, TGF-β treatment caused to an increase in plasminogen activator inhibitor type 1 transcriptional activity through pSmad3L (11). Because plasminogen activator inhibitor type 1 conducts the cells to migration and invasion by blocking cellular adhesion and by promoting basement membrane degradation, stimulation of plasminogen activator inhibitor type 1 production might lead to an increase in invasive capacity of cancer cells (32). In support of this role of pSmad3L, late-stage cancer with deep invasion and/or metastasis typically was characterized by high phosphorylation of Smad3L. Collectively, the change of phosphorylation sites in Smad2/3 from COOH-terminal regions to linker regions is one of the major mechanisms for the complex transition of TGF-β signaling during human colorectal carcinogenesis.

Few pSmad2/3L-positive epithelial cells could be seen in normal epithelial crypts. Adenomas showed degree of linker phosphorylation that was a few times greater than in normal mucosa (i.e., a pSmad2/3L signal intensity intermediate between those typical of normal epithelial cells and adenocarcinomas). Taken together with...
the high degree of pSmad2/3L in primary invasive adenocarcinoma, our results suggest that the linker phosphorylation develops relatively late in the evolution of colorectal tumors accompanied by persistent JNK activity.

Because K-ras mutations have been detected in both adenoma and carcinoma (1) and JNK can be activated through ras (33), increased JNK-dependent phosphorylation of Smad2/3L observed in colorectal tumors may be a direct consequence of the K-ras mutation. However, the degree of the linker phosphorylation in individual tumors did not reflect the presence of K-ras mutation. Thus, only 47% of tumors studied harbored mutations of K-ras (data not shown), whereas all adenomas and adenocarcinomas examined displayed elevated JNK-dependent phosphorylation of Smad2/3L. Several pathways including Src are responsible for activation of JNK during colorectal carcinogenesis (34, 35).

Because TGF-β signaling participates in the physiology of normal epithelial cells, unraveling the molecular mechanisms of TGF-β signaling leading to pathogenesis of human cancer is critical to development of new therapies (36, 37). In addition to continued molecular studies in vitro, investigations need to be extended to more complex in vivo models (2). The present work represents such application of studies on TGF-β signaling in vitro to one in vivo situation, resected human tissues. In the process of human colorectal carcinogenesis, pSmad3C, which favors tumor suppressor activity of TGF-β, was found to decrease, whereas JNK tended to induce the phosphorylation of Smad2/3L. From the view point of TGF-β signaling, a key therapeutic challenge in cancer would be restoration of the lost tumor suppressor function observed in normal colorectal epithelial cells at the expense of oncogenic effects that would lead to more aggressive adenocarcinoma (36). Specific inhibitors of the JNK-mediated Smad2/3L pathway might prove useful in this respect. Investigation concerning the distribution of pSmad2/3L in a given specimen would be needed to predict potential response to molecularly targeted therapy for human sporadic colorectal cancer.

Acknowledgments

Received 6/22/04; revised 10/4/04; accepted 10/27/04.

Grant support: Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. R. Derynck (University of California at San Francisco, San Francisco, CA) for providing the cDNAs encoding human Smad2 and Smad3.

References

4. Heldin CH, Miyazono K, Ten Dijke P. TGF-β and Smad Signaling via JNK in Colorectal Cancer...
Acceleration of Smad2 and Smad3 Phosphorylation via c-Jun NH₂-Terminal Kinase during Human Colorectal Carcinogenesis

Hideo Yamagata, Koichi Matsuzaki, Shigeo Mori, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/1/157

Cited articles
This article cites 35 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/1/157.full#ref-list-1

Citing articles
This article has been cited by 24 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/1/157.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.