Smad4 and Transforming Growth Factor β1 Expression in Patients with Squamous Cell Carcinoma of the Esophagus

Shoji Natsugoe,2 Che Xiangming, Masataka Matsumoto, Hiroshi Okumura, Saburo Nakashima, Hironori Sakita, Sumiya Ishigami, Masamichi Baba, Sonshin Takao, and Takashi Aikou

First Department of Surgery, School of Medicine, Kagoshima University, Kagoshima 890-8520, Japan

ABSTRACT

Purpose: The members of the Smad family play key roles in regulating gene expression in the transforming growth factor (TGF)-β1 signaling pathways. Activation of Smads causes their translocation from the cytoplasm to the nucleus, where they function as transcription factors. The present study analyzed the expression and clinicopathological significance of Smad4 and TGF-β1 in squamous cell carcinoma of the esophagus.

Experimental Design: Immunohistochemistry was used to investigate the expression of Smad4 and TGF-β1 proteins in 258 patients with squamous cell carcinoma of the esophagus. The relationship between expression of these proteins and clinicopathological factors was analyzed, and the usefulness of Smad4 in disease prognosis was evaluated in relation to TGF-β1 expression.

Results: Smad4 expression was preserved in 32.2% of tumors, and TGF-β1 expression was identified in 42.6% of tumors. Patients with preserved expression of Smad4 had a higher rate of early-stage carcinoma (P < 0.01) and fewer lymph node metastases (P < 0.01) than those with reduced Smad4 expression. The expression of TGF-β1 was not associated with any of the clinicopathological factors. Postoperative survival analysis indicated that patients with a tumor in which Smad4 expression was reduced had worse clinical outcomes than those with preserved expression (P = 0.01). In patients with TGF-β1-negative tumors, the survival rate was significantly higher in patients with a preserved level of Smad4 expression than in those with reduced Smad4 expression (P = 0.02). However, according to multivariate analysis, Smad4 expression could not be used as an independent prognostic factor.

Conclusions: Although Smad4 expression could not be used as a prognostic factor, its expression reflected tumor progression such as tumor depth and lymph node metastasis.

INTRODUCTION

The Smad protein family is a family of intracellular signal transducers that act downstream of receptors for TGF3 family members (1). Cell cycle progression of normal epithelial cells is inhibited by exogenous TGF, whereas malignant epithelial cells are often resistant to the growth-inhibitory effects of TGF (2). In some epithelial malignancies, TGF resistance is associated with functional inactivation of either TGF receptors (3) or signal transducers of the Smad family (4), suggesting that resistance to TGF-induced growth inhibition is attributable to disrupted TGF/Smad-dependent regulation of transcription. On the basis of its role in mediating the growth-inhibitory effects of TGF in normal cells and its loss of function in some tumor types, Smad4 is believed to be a tumor suppressor protein (5).

Smad4 is apparently common to all of the ligand-specific Smad pathways and appears to play a role as a central mediator in TGF superfamily signaling (6, 7). Smad4 appears to mediate the actions of other Smad proteins. In vitro and in vivo studies have indicated that a mutation in the Smad4 gene, on chromosome 18q21, plays a significant role in the tumorigenesis and progression of carcinoma of the pancreas, colorectum, stomach, and other solid tumors (8–15). Although mutation of the Smad4 gene is a rare event in esophageal carcinoma (16), any relationship between Smad4 protein expression and clinicopathological features remains unclear.

The overexpression of TGF-β1 has been reported in tissues from various types of carcinomas (17–19). TGF-β1 expression appears to correlate with clinicopathological findings, especially with the prognosis of cancer patients (20–23). However, the role of Smad4 remains unclear in squamous cell carcinoma of the esophagus. The purpose of this study was to elucidate the clinical significance of Smad4 and its correlation with TGF-β1 expression in esophageal squamous cell carcinoma.

MATERIALS AND METHODS

Patients and Specimens. Between 1987 and 1998, 421 patients were admitted to Kagoshima University Hospital. Of these patients, 35 patients with endoscopic mucosal resection, 34 patients with palliative resection, 40 patients with preoperative chemotherapy and/or radiotherapy, and 54 patients with synchronous or metachronous multiple cancer in other organs were excluded from this study. Thus, 258 consecutive patients...
with esophageal carcinoma who underwent esophagectomy with lymph node dissection were enrolled in the current study. The patients, 243 males and 15 females, ranged in age from 38 to 84 years (mean, 63.8 years) and had not undergone preoperative radiotherapy or chemotherapy. All patients were followed up after discharge by X-ray examination and tumor marker studies (squamous cell carcinoma antigen and carcinoembryonic antigen) every 1–3 months, computed tomography every 3–6 months, and ultrasonography every 6 months. Bronchoscopic and endoscopic examinations were performed when necessary. The follow-up data after surgery were obtained from all patients with a median follow-up period of 26 months (range, 2–164 months).

On the basis of the tumor-node-metastasis (TNM) classification of the International Union against Cancer (24), 45 of the 258 patients had $T_1$ tumors, 34 patients had $T_2$ tumors, 119 patients had $T_3$ tumors, and 60 patients had $T_4$ tumors. With regard to location, 33 tumors were located in the upper third of the esophagus, 134 tumors were located in the middle third of the esophagus, and 91 tumors were located in the lower third of the esophagus. Pathologically, all of the tumors were squamous cell carcinoma (101 tumors were well differentiated, 113 tumors were moderately differentiated, and 44 tumors were poorly differentiated). Lymph node metastases were present in 165 of 258 patients (64.0%). All of the $M_1$ tumors were attributable to distant lymph node metastases.

Immunohistochemical Staining and Evaluation. The specimens were cut into 3-μm-thick sections and mounted on glass slides. Immunohistochemical staining was performed using avidin-biotin peroxidase method as described previously (22, 23, 25). Briefly, after deparaffinizing in xylene and rehydrating in ethanol, the sections were heated in a citrate buffer (0.01 M, pH 6.5) at 120°C for 10 min to reveal the antigen. Next, sections were incubated with either the primary anti-Smad4 monoclonal antibody (1:300; Smad4: B-8; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-TGF-β1 antibody (1:100; TGF-β1: V; Santa Cruz Biotechnology, Inc.) and left overnight at 4°C. The sections were incubated with biotinylated antimouse IgG and avidin-biotin peroxidase (Vector Laboratories, Burlingame, CA) and visualized using diaminobenzidine tetrahydrochloride. In the negative control group, 1% BSA was used in place of the primary antibody.

The expression of Smad4 by malignant cells was compared with that of normal epithelial cells located away from the tumor. Tumor cells that stained as strongly as normal epithelial cells were considered positive (+), whereas those that showed weaker staining patterns than normal epithelial cells or did not stain at all were considered weak (±) or negative (−), respectively. The tumors were then classified based on their Smad4 expression after overview of the section and were considered to have preserved Smad4 expression if >50% of the tumor cells were Smad4 positive. The tumors classified as having reduced expression were those that did not fit into the above categories.

TGF-β1-positive expression was determined by counting the number of tumor cells in which the cytoplasm was stained with the anti-TGF-β1 antibody. To evaluate this, 10 fields (within the tumor and at the invasive front) were selected, and expression in 1000 tumor cells (100 cells/field) was evaluated using high power (×200) microscopy. The average labeling index of TGF-β1 was assessed according to the proportion of positive cells in each field. TGF-β1 expression was graded as negative (−) if ≤10% of cancer cells were stained or positive (+) if >10% of cancer cells were stained (21, 22).

Statistical Analysis. A statistical analysis of group differences was performed using $\chi^2$ and $t$ tests. The Kaplan-Meier method was used for survival analysis and evaluated by the Wilcoxon test. The prognostic factors were examined by univa-
riate and multivariate analyses (proportional hazards regression model). \( P < 0.05 \) was considered statistically significant.

RESULTS

Expression of Smad4 and TGF-\( \beta \)-1. In the majority of cancer cells, Smad4-positive staining was observed in the cytoplasm and simultaneously in the nuclei of some cancer cells. All normal epithelial cells were Smad4 positive, whereas only 32.2\% of esophageal cancerous tissue showed preserved Smad4 expression (Fig. 1A), with 67.8\% showing reduced expression (Fig. 1B).

The TGF-\( \beta \) protein was found mainly in the cytoplasm of cancer cells (Fig. 1C) and was occasionally evident in fibroblasts and smooth muscle cells. The positive tumor cells were distributed heterogeneously throughout the tumor. The TGF-\( \beta \) protein was identified in 42.6\% (110 of 258 patients) of the tumors analyzed.

Smad4 or TGF-\( \beta \)-1 Expression and Clinicopathological Features. The number of cells with reduced Smad4 expression increased as the tumors invaded deeper layers (\( P < 0.01 \)). The percentage of lymph node metastasis was significantly different in patients with preserved Smad4 expression compared with patients with reduced Smad4 expression (\( P < 0.01 \)). Further analysis revealed that the patients with preserved Smad4 expression had early-stage esophageal carcinoma. The expression of TGF-\( \beta \)-1 was not associated with any of the clinicopathological factors (Table 1).

Clinical Outcome and the Expression of Smad4 or TGF-\( \beta \)-1. There appeared to be a correlation between Smad4 expression and clinical outcome: the 5-year survival rate of patients with tumors in which Smad4 expression was preserved was 37.7\%, whereas the survival rate of patients with tumors in which Smad4 expression was reduced fell to 23.1\%. There was a significant difference in 5-year survival between preserved and reduced expression of Smad4 (\( P = 0.01 \); Fig. 2A). However, there did not appear to be any correlation between 5-year survival rates and TGF-\( \beta \)-1 expression (Fig. 2B). The survival rate of patients with preserved and reduced expression of Smad4 was further analyzed to see whether there was any relationship between Smad4 and TGF-\( \beta \)-1 expression. In patients with TGF-\( \beta \)-1-positive tumors, there was no significant difference in 5-year survival rates between patients with preserved and reduced expression of Smad4 (Fig. 3A). However, in patients with TGF-\( \beta \)-1-negative tumors, the survival rate was significantly higher in patients with preserved Smad4 expression than in patients with reduced Smad4 expression (\( P = 0.02 \); Fig. 3B).

The factors relating to patient prognosis were evaluated by univariate and multivariate analyses (Table 2). The univariate analysis showed that age, gender, depth of invasion,
lymph node metastasis, stage, lymphatic invasion, venous invasion, and Smad4 expression were related to postoperative survival ($P/1100.05$). However, according to multivariate regression analysis, patient age, gender, depth of invasion, stage, and venous invasion were independent prognostic factors, but Smad4 expression was not an independent prognostic factor.

**DISCUSSION**

Immunostaining revealed that Smad4 is localized in both the cytoplasm and nuclei as reported in cases of carcinoma of the colon and stomach (25, 26). Although mutation of the Smad4 gene was reportedly rare in esophageal carcinoma (16), 67.8% of esophageal cancerous tissue showed reduced Smad4 expression in the present study. These results suggest that loss of heterozygosity or the process of mRNA translation may be related to reduced expression of Smad4 in esophageal carcinoma. Our study suggests that Smad4 expression decreases from the superficial layer to the deeper layer in the same tumor. Furthermore, Smad4 expression is preserved in early-stage carcinoma, compared with advanced carcinoma, and is related to the depth of tumor invasion and lymph node metastasis. In addition, our results suggest that there is a relationship between Smad4 expression and progression of esophageal carcinoma. However, there does not appear to be any relationship between TGF-β1 and clinicopathological findings. TGF-β1 expression is found irrespective of the depth of tumor invasion or lymph node metastasis. It is suggested that ~50% of patients with esophageal squamous cell carcinoma have an abnormality in the TGF-β1 protein level, irrespective of the stage of tumor progression.

The present study indicates that the 5-year survival rate is significantly higher in patients with preserved Smad4 expression than in those with reduced Smad4 expression because patients with preserved expression belong to a group of patients with relatively early-stage carcinoma. To better understand the role of Smad4 and its relationship to the TGF-β1 superfamily, we also studied TGF-β1 expression. Our results suggest that the 5-year survival rate does not differ significantly according to

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*Fig. 2* The postoperative survival curve of patients according to their expression of Smad4 (A) or TGF-β1 (B).

*Fig. 3* The survival rate did not differ in TGF-β1-positive tumors (A). The postoperative survival rates of patients with TGF-β1-negative tumors and with preserved (Pre) or reduced (Red) Smad4 expression were significantly different. The patients with preserved Smad4 expression (Pre) had a better outcome than those with reduced Smad4 expression (Red; $P = 0.02$; B).

**Table 2** Univariate and multivariate analyses of prognostic factors in esophageal cancer

<table>
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<th>Univariate Hazard ratio</th>
<th>Multivariate Hazard ratio</th>
<th>95% Confidence interval</th>
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<td>Age</td>
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<td>Gender</td>
<td>0.0171</td>
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<td>Histology</td>
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<td>pT</td>
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<td>pN</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
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<td>2.083</td>
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<td>Lymphatic invasion</td>
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<td>Venous invasion</td>
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<td>Smad4</td>
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<tr>
<td>TGF-β1</td>
<td>0.9202</td>
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</table>
TGF-β1 expression between patients whose tumors express TGF-β1 and patients whose tumors do not express TGF-β1. Several studies suggest that there is a relationship between TGF-β1 expression and the prognosis of patients with various types of carcinomas (20–23, 26). However, few reports have been published on the relationship between TGF-β1 expression and prognosis of patients with esophageal squamous cell carcinoma. The results obtained from our study suggest that there is a relationship between TGF-β1 expression and tumor carcinogenesis, but not tumor progression, in esophageal squamous cell carcinoma.

Because TGF-β1 acts as a cell cycle inhibitor, which has a biphasic effect on tumor growth, some factor in turn must influence TGF-β1 activity. In the present study, we therefore analyzed the presence of both TGF-β1 and Smad4 in tumor cells. The results indicate that patients with TGF-β1-negative tumors have significantly higher postoperative survival rates when Smad4 expression is preserved. This reflects both the effects of TGF-β1 on carcinogenesis of esophageal squamous cell carcinoma and the relationship between TGF-β1 and its signaling transducer, Smad4. The correlation between TGF-β1 and Smad4 has an influence on the prognosis of esophageal cancer patients. However, multivariate analysis suggests that Smad4 expression is not an independent prognostic factor but is closely related to tumor depth or lymph node metastasis.

In conclusion, Smad4 expression is related to the stage of tumor progression such as depth of invasion and lymph node metastasis. Although Smad4 is not an independent prognostic factor, the examination of Smad4 expression, especially its relationship to the TGF-β superfamily signaling pathway, is useful for gaining a better understanding of the malignant property of esophageal squamous cell carcinoma.

REFERENCES

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