Prognostic significance of the expression of Smad4 and Smad7 in human gastric carcinomas

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Background: Transforming growth factor-β (TGF-β) modulates the growth and function of many cells, including those with malignant transformation. Smad proteins have been identified as major components in the intracellular signaling of TGF-β family members.

Patients and methods: To clarify the correlations between clinicopathologic profiles and the patient’s survival, the expression of common mediator Smad (Smad4) and inhibitory Smad (Smad7) were evaluated immunohistochemically in 304 consecutive gastric carcinomas using the tissue array method.

Results: Positive Smad4 expression was observed in 266 (87.5%) tumors and positive Smad7 expression in 98 (32.2%) tumors. The prognosis of patients with a Smad4-positive tumor was significantly better than that of the patients with a negative tumor. The survival rate was significantly higher in patients with negative Smad7 expression than those with positive Smad7 expression. In subgroup analysis according to TNM (tumour–node–metastasis) stage, both Smad4 and Smad7 showed most significant prognostic differences in stage I gastric cancer patients. Multivariate analysis indicated that tumor size, depth of invasion, lymph node metastasis and Smad7 expression were independent prognostic factors.

Conclusion: Enhanced expression of the TGF-β signaling inhibitor Smad7 may present one of the novel mechanisms of TGF-β resistance in human gastric carcinomas.

Key words: gastric carcinoma, prognostic factor, Smad4, Smad7, transforming growth factor-β

Introduction

Deregulated transforming growth factor-β (TGF-β) family signaling has been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have revealed that Smad proteins, discovered through genetic immune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1]. Recent studies have been implicated in various human diseases, including autoimmune diseases, vascular disorders and cancer [1].

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The purpose of this study was to investigate the expression of Smad4 and Smad7 proteins and their prognostic significance in human gastric carcinoma.

Materials and methods

Patients

A total of 304 gastric carcinoma patients who had undergone gastrectomy at Seoul National University Hospital from 1 January 1995 to 30 June 1995 were included in this study. None of the patients had received prior chemotherapy or irradiation therapy. All patients had histologically proven adenocarcinoma of the stomach. The clinicopathologic findings were determined according to the criteria established by the Japanese General Rules for Gastric Cancer Study. There were 203 male patients and 101 female patients, and their ages ranged from 21 to 80 years (mean 54.8 years). The clinicopathological characteristics of the patients are given in Table 1. Among them, most patients underwent gastrectomy with UICC [International Union Against Cancer (Union Internationale Contre le Cancer)] R0 category [n = 286 (92.8%)], followed by R2 [n = 16 (5.3%)] and R1 categories [n = 6 (2.0%)]. We selected the following 12 prognostic factors for evaluation: age, sex, tumor size, tumor location, Lauren histology, differentiation, depth of invasion, lymph node metastasis, distant metastasis, stage, lymphatic invasion and vascular invasion. Survival data were available for all patients and obtained from patient records and the Population Registry in Korea. During follow-up, 110 (36.2%) patients died of gastric cancer. The overall 5-year survival rate was 62.6% and the median follow-up was 67 months (range 1–72 months).

Tissue array methods

Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric tumors (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). Each tissue array block contained up to 60 cases, allowing six array blocks to contain the total 304 cases. An adequate case was defined as tumor occupying >10% of core area. Each block contained an internal control consisting of non-neoplastic gastric mucosa. Sections of 4 µm were cut from each tissue array block, deparaffinized and dehydrated.

Immunohistochemistry

Immunohistochemical staining against Smad4 [1:100; B-8, sc-7966 (Santa Cruz Biotechnology, Santa Cruz, CA)] and Smad7 [1:100; H-79, sc-11392 (Santa Cruz Biotechnology)] was performed using a streptavidin peroxidase procedure. After deparaffinization and rehydration, tissue sections were treated three times with microwaves in 0.01 M citrate buffer (pH 6.0) for 5 min each time. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 6 min to block the endogenous peroxidase activity, and incubated in 2.5% blocking serum to reduce non-specific binding. After incubation with primary antibodies, the sections were incubated with biotinylated anti-rabbit IgG and avidin–biotin peroxidase (Vector Laboratories, Burlingame, CA), and visualized using diaminobenzidine tetrahydrochloride. Two antibodies among various commercially available antibodies were selected after the test procedure using a human control slide for immunohistochemistry (Superbiochips Laboratories).

For statistical analysis, the results of immunostaining were considered to be positive if ≥10% of the neoplastic cells were stained. Previous studies in which immunolabeling patterns have been correlated with Smad4 gene status have shown that both focal and diffusely positive labelings correlate with an intact Smad gene, whereas complete loss of labeling correlates with inactivation of the Smad gene [22]. On the contrary, there have been few studies dealing with the definite level of Smad7 positivity in immunohistochemical staining, so we applied the previously reported methodology on Smad7 positivity in immunohistochemical staining [23]. For purposes of data analysis, both focal and diffusely positive lesions were considered to show intact Smad expression (positive), and only complete loss of labeling was considered to show loss of Smad expression (negative).

Statistical analysis

The association of factors was evaluated using the chi-square test. The significance of differences among means was determined by the Mann–Whitney U-test. Survival rates were calculated using the Kaplan–Meier method and analyzed using the Log-rank test. A multivariate analysis was performed using the Cox proportional hazards model. The significance level was set at 5% for all analyses. All statistical analyses were conducted using the SPSS 10.0 statistical software program (SPSS, Chicago, IL).

Results

Immunohistochemical staining for Smad4 and Smad7

Smad4 protein was consistently expressed in a nuclear location (Figure 1A and B). Smad4 was homogeneously stained in normal cells but the rates of Smad4 expression were reduced in gastric cancer tissues. Smad7 protein was stained mainly in the cytoplasm of cancer cells (Figure 1C and D). Smad7 overexpression was observed in gastric cancer tissues, whereas no expression was observed in normal tissues.

Relationship between Smad4 or Smad7 expression and clinicopathologic findings

Positive Smad4 and Smad7 expression was observed in 266 (87.5%) and 98 (32.2%) tumors, respectively. The correlation between Smad4 or Smad7 expression and the clinicopathologic findings are shown in Table 1. The rate of positive Smad4 expression was higher in female patients, smaller tumor sizes, undifferentiated tumors and diffuse tumor type than in male patients, larger tumor sizes, differentiated tumors and intestinal tumor type (P <0.05). However, the rate of Smad4-positive expression decreased as tumors invaded deeper layers or in tumors with more advanced stages (P <0.05). The rate of Smad7-positive expression was significantly higher in the patients with differentiated tumors or intestinal type of tumors than in those with undifferentiated tumors or diffuse tumor type (P <0.05). There was no significant correlation between Smad7 expression and sex, tumor size, vascular invasion, lymphatic invasion, depth of invasion, lymph node metastasis or clinical stage.

Correlation between Smad4 or Smad7 expression and survival rate

The 5-year survival rate was 64.4% in patients with Smad4-positive tumors and 49.8% in patients with Smad4-negative tumors. Accordingly, the prognosis for patients with a Smad4-positive tumor was significantly better than for patients with a negative tumor (P = 0.017). In subgroup analysis according to TNM (tumor–node–metastasis) stage [24], the survival rate of patients with a Smad4-positive tumor was significantly higher than that of patients with a Smad4-negative tumor with stage I (93.3% versus 77.9%; P = 0.037) and IV (21.6% versus 0%; P = 0.05), but there
were no statistical significances in the tumors with stage II (65.3% versus 87.5%) and III (40.7% versus 45.5%) (Figure 2). Moreover, the 5-year survival rate of patients with tumors in which Smad7 expression was positive was 52.2%, whereas the survival rate of patients with tumors in which Smad7 expression was negative was 67.5%. The survival rate was significantly higher in patients with negative Smad7 expression than in patients with positive Smad7 expression ($P = 0.011$). In subgroup analysis according to TNM stage, the survival rate of patients with a Smad7-negative tumor was significantly higher than that of patients with a Smad7-positive tumor with stage I (95.6% versus 84.1%, $P = 0.025$) and III (50.0% versus 26.1%, $P = 0.05$), but there were no statistical significances in the tumors with stage II (66.6% versus 72.2%) and IV (17.2% versus 16.7%) (Figure 3).

The factors relating to patient prognosis were evaluated by univariate and multivariate analyses. Univariate analysis showed that tumor size, Lauren histology, differentiation, depth of invasion, lymph node metastasis, distant metastasis, lymphatic invasion, vascular invasion, UICC R category, Smad4 expression and Smad7 expression were related to survival rate ($P < 0.05$). In the multivariate Cox model, tumor size, depth of invasion, lymph node metastasis and Smad7 expression were independent prognostic factors for cancer-specific survival (Table 2).

Co-expression of Smad4 and Smad7, and its prognostic significance

The numbers of tumors with Smad4$^-$/Smad7$^-$, Smad4$^+$/Smad7$^-$, Smad4$^-$/Smad7$^+$ and Smad4$^+$/Smad7$^+$ expression were 20 (6.6%), 186 (61.2%), 18 (5.9%) and 80 (26.3%), respectively. The expressions of Smad4 and Smad7 were inversely correlated with each other ($P = 0.04$). The 5-year survival rate for patients with Smad4$^+/Smad7^-$ expression was most favorable (70.3%), while that with Smad4$^-/Smad7^+$ expression was most unfavorable (38.9%), which showed a significant difference ($P = 0.001$). The 5-year survival rates with Smad4$^-/Smad7^-$ and with Smad4$^+/Smad7^+$ expression were found to lie in between (59.4% and 59.6%, respectively) (Figure 4).

Discussion

Downstream events in the TGF-β signaling pathway include complex formation of Smad2 or Smad3 with Smad4, translocation of
the Smad2,3/Smad4 complex to the nucleus, and eventual activation of target genes [25]. In the absence of ligand, the inhibitory Smads, Smad6 and Smad7, are localized predominantly in the nucleus [26]. Upon TGF-β receptor activation, they accumulate in the cytoplasm and associate with the ligand-activated TGF-β receptor complex in the cell membrane, antagonizing TGF-β family signaling by preventing the activation of signal-transducing Smad complexes.

In this study, we examined Smad4 and Smad7 expression in gastric carcinomas to elucidate their role in tumor progression. The tissue array method was used to analyze Smad4 and Smad7 proteins in 304 consecutive gastric carcinomas. The tissue array method enabled us to analyze a large number of gastric carcinomas, and consecutive sections from the array blocks allowed different protein expressions to be analyzed from defined, almost morphologically identical, tumor regions.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Smad4 expression (%)</th>
<th>Smad7 expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 266)</td>
<td>Negative (n = 38)</td>
</tr>
<tr>
<td>Age</td>
<td>54.7 ± 12.8</td>
<td>55.3 ± 13.4</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>172 (84.7)</td>
<td>31 (15.3)</td>
</tr>
<tr>
<td>Female</td>
<td>94 (93.1)</td>
<td>7 (6.9)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>5.0 ± 3.0</td>
<td>6.5 ± 2.6</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiated</td>
<td>91 (81.3)</td>
<td>21 (18.8)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>175 (91.1)</td>
<td>17 (8.9)</td>
</tr>
<tr>
<td>Lauren type</td>
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<tr>
<td>Intestinal</td>
<td>94 (81.0)</td>
<td>22 (19.0)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>145 (91.2)</td>
<td>14 (8.8)</td>
</tr>
<tr>
<td>Vascular invasion</td>
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<td></td>
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<tr>
<td>Absent</td>
<td>253 (88.2)</td>
<td>34 (11.8)</td>
</tr>
<tr>
<td>Present</td>
<td>13 (76.5)</td>
<td>4 (23.5)</td>
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<tr>
<td>Lymphatic invasion</td>
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<td></td>
</tr>
<tr>
<td>Absent</td>
<td>198 (89.6)</td>
<td>23 (10.4)</td>
</tr>
<tr>
<td>Present</td>
<td>68 (81.9)</td>
<td>15 (18.1)</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
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</tr>
<tr>
<td>T1</td>
<td>92 (98.9)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>T2</td>
<td>120 (84.0)</td>
<td>23 (16.0)</td>
</tr>
<tr>
<td>T3</td>
<td>49 (77.6)</td>
<td>14 (22.4)</td>
</tr>
<tr>
<td>T4</td>
<td>5 (100.0)</td>
<td>0 (0.0)</td>
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<tr>
<td>Lymph node metastasis</td>
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<tr>
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<td>105 (91.3)</td>
<td>10 (8.7)</td>
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<tr>
<td>Present</td>
<td>161 (85.2)</td>
<td>28 (14.8)</td>
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<tr>
<td>Stage</td>
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<tr>
<td>I</td>
<td>123 (93.2)</td>
<td>9 (6.8)</td>
</tr>
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<td>II</td>
<td>52 (86.7)</td>
<td>8 (13.3)</td>
</tr>
<tr>
<td>III</td>
<td>54 (83.1)</td>
<td>11 (16.9)</td>
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<tr>
<td>IV</td>
<td>37 (78.7)</td>
<td>10 (21.3)</td>
</tr>
</tbody>
</table>

NS, not significant.

Table 1. The relationship between Smad4 or Smad7 expression and clinicopathologic findings in gastric carcinomas

The Smad4 gene, located at 18q21, has been found to undergo frequent alteration in pancreatic cancers [7] and LOH studies have suggested that a gene on chromosome 18q is altered frequently in intestinal-type gastric carcinomas [27]. In our study, the loss of Smad4 protein expression was also statistically significantly associated with intestinal type, and associated with male sex, larger tumor size, differentiated tumors, deep penetration, advanced clinical stage and patient survival. Xiangming et al. reported that the reduced expression of Smad4 was related to the depth of tumor invasion and that Smad4 was an independent prognostic factor. Therefore, mutation in the Smad4 gene and loss of Smad4 protein either resulted in tumorigenesis or was associated with worse patient survival.
with malignancy and progression of tumors [17]. Our results also revealed that the loss of Smad4 expression was related to the deep penetration and poor survival, but Smad4 was not an independent prognostic factor in multivariate analysis in our series [28]. These different results may be attributable to the selection of patients and different cut-off positive value, or to the evaluation of the results.

Smad7 inhibits TGF-β-induced transcriptional responses [29]. Smad7 associates with the activated TGF-β receptor and interferes with the activation of Smad2 and Smad3 by preventing their receptor interaction and phosphorylation. It has been reported that Smad7 acts as an important molecule for regulating TGF-β activity in human disease. Monteleone et al. reported that Smad7 was overexpressed in irritable bowel disease mucosa and purified mucosal T cells [30]. In a separate study, Kleeff et al. reported that Smad7 enhances tumorigenicity in pancreatic cancer [19]. Using in vitro and in vivo studies, they revealed that pancreatic cancer cells have redundant barriers to TGF-β signaling that may allow the cancer cells to escape TGF-induced growth inhibition, while still allowing for the expression of metastasis-promoting genes such as PAI-1. But, until recently, whether Smad7 expression is associated with clinicopathological parameters such as tumor stage and prognosis has not been reported. In this study, Smad7 expression was significantly more frequent in intestinal type and differentiated carcinoma. The underlying etiology of this tumor heterogeneity is not well understood. The survival time analysis revealed a significant correlation between Smad7 expression and length of disease-free survival. The prognostic value of Smad7 was independent of other well established clinical prognostic factors such as depth of invasion or nodal involvement. We conclude that Smad7 plays an important role in the development of gastric carcinoma and that overexpression of Smad7 may be a significant independent prognostic indicator for clinical outcome in patients with gastric carcinoma.

In subgroup analysis according to TNM stage, although Smad4 (stage IV) and Smad7 (stage III) showed some marginal prognostic significance, both Smad4 and Smad7 showed the most significant prognostic differences only in stage I gastric cancer patients. The reason why Smad4 and Smad7 expression should be most prognostic only in stage I patients is unclear, but in case of Smad4-negative stage I gastric cancer patients, all patients were
classified as having stage Ib disease (eight T2N0M0 and one T1N1M0). In the clinical setting, this result could be applied very usefully for selecting the patients who should be followed closely and considered as candidates for adjuvant treatment among patients with stage I gastric cancer.

As mentioned previously, Smad4 and Smad7 are both located on chromosome 18q21. Therefore, it would be of considerable interest to know whether gene expression of Smad4 and Smad7 were independent of each other. However, our results showed that the expression patterns of Smad4 and Smad7 were inversely correlated with each other ($P = 0.04$), which suggests that these two closely located genes might be expressed by a different mechanism.

As expected, comparing co-expression of Smad4 and Smad7 with survival, the 5-year survival rate for patients with Smad4+/Smad7+ expression was most favorable, while that with Smad4+/Smad7– expression was most unfavorable [significant difference ($P = 0.001$)]. The 5-year survival rates with Smad4+/Smad7+ and Smad4+/Smad7– expression were found to lie in between. Therefore, evaluating the expression profile of both Smad4 and Smad7 together could be a more useful prognostic marker for gastric cancer patients than evaluating only one expression profile of either Smad4 or Smad7.

In summary, this study has demonstrated that Smad7 expression is associated with poor outcome in gastric carcinomas. These observations further underscore the importance of Smad proteins in carcinogenesis, and indicate that Smad7 expression may present one of the novel mechanisms for TGF-β resistance in human gastric carcinoma. As both Smad4 and Smad7 showed most significant prognostic differences in stage I gastric cancer patients, Smad expression in gastric cancer could be useful in selecting the patients who should be closely followed and considered as candidates for adjuvant treatment among those with stage I gastric cancer.

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### References


