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ORIGINAL ARTICLE

A study of Smad4 and Smad7 expression in surgically resected samples of gastric adenocarcinoma and their correlation with clinicopathological parameters and patient survival

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Summary

Purpose: *The canonical signaling pathway for the transforming growth factor-beta (TGF-β) family is through the Smad proteins which are pivotal intracellular mediators of TGF-β family members. Recently, disruption of the TGF-β pathway in cancer has been demonstrated at the level of the Smad signal transducers. In this study, we examined Smad4 and Smad7 expression in gastric carcinomas to elucidate their role in tumor progression.*

Methods: *The immunohistochemical expression of Smad4 and Smad7 was evaluated in 151 surgically resected samples of gastric adenocarcinoma in order to examine their correlation with clinicopathologic findings and patients' survival.*

Results: *Smad4 and Smad7 expression (low, moderate or strong) was observed in 86.7% (131/151) and 33.1% (50/151) of gastric adenocarcinoma tumor samples, respectively. Our results revealed that the loss of Smad4 expression correlated significantly with the intestinal type, male sex, depth of tumor and poor survival. Smad7 expression was significantly more frequent in intestinal type and well differentiated gastric adenocarcinomas and significantly correlated with the duration of disease-free survival.*

Conclusion: *Smad signal transducers are considered as important molecules in tumor development and progression and the evaluation of their expression in human gastric cancer could be useful in selecting stage I patients who should be considered as candidates for adjuvant chemotherapy.*

Key words: *gastric adenocarcinoma, prognosis, Smad4, Smad7, stomach*

Introduction

TGF-β signaling is one of the most important tumor suppressor pathways [1]. Recent studies have revealed that Smad proteins, discovered through genetic studies in Drosophila and Caenorhabditis elegans, are pivotal intracellular mediators of TFG-β family members [2,3]. According to their specific functions Smads can be classified into the receptor-regulated Smads (R-Smads: Smad1, 2, 3, 5 and 8), common mediator Smads (Co-Smads: Smad4) and inhibitory Smads (I-Smads: Smad6 and 7) [4,5]. The demonstration that TGF-β has antiproliferative effects in a variety of cell types has led to the hypothesis that inactivation of the TGF-β signaling pathway contributes to tumor development or progression [6]. Recently, disruption of the TGF-β pathway in cancer has been demonstrated at the level of the Smad signal transducers [7].

Loss of Smad4 expression is a common feature of most human malignancies. The gene encoding Smad4 was originally cloned as a tumor suppressor gene on chromosome 18q21, which is frequently deleted or mutated in pancreatic carcinomas. Hence, its original name was DPC4 (deleted in pancreatic carcinoma locus 4) [8]. Smad4 mutations have also been observed in a significant proportion of colorectal tumors and less frequently in breast, ovarian, head and neck, prostatic and esophageal tumors [9-13].

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The inhibitory Smads, Smad6 and Smad7 have been shown to bind to the TGF-β type I receptor, precluding the phosphorylation of the receptor-regulated Smads, and consequently, serving as an endogenous negative feedback system to receptor TGF-β signaling [14].It has been suggested that Smad7 may induce tumorigenicity by blocking TGF-β-induced growth inhibition and apoptosis. The position of the gene for Smad7 has been assigned to the region 18q21, identical to Smad4, by *in situ* hybridization and mapped between Smad2 and Smad4 genes with a 4-Mb gene cluster [15,16]. The expression of Smad7 is very low in epithelial tissues, but is upregulated in several cancers, such as pancreatic and colon cancer. However, little is known about the roles of Smad7 in gastric carcinoma.

In this study, we aimed to further establish the role of Smad4 as a potential prognostic marker for gastric adenocarcinoma and to clarify the role of Smad7 in gastric cancer biology. We finally determined the relationship of Smad4 and Smad7 with certain clinicopathological parameters of the tumor.

Methods

Patients

A total of 151 gastric carcinoma patients who had undergone gastrectomy at the First Department of Surgery, Tzaneio General Hospital, Piraeus, Greece, from 1 January 2002 to 31 December 2009 were included in this study. None of the patients had received neoadjuvant chemotherapy or preoperative radiotherapy. All patients had histologically proven adenocarcinoma of the stomach. There were 87 male patients and 64 female patients, and their ages ranged from 25 to 81 years (mean 56.8). Clinical and pathological data were obtained from the patients' medical records. We selected the following 8 prognostic factors for evaluation: age, sex, depth of invasion, Lauren histology, differentiation, lymph node metastasis, vascular invasion and lymphatic invasion. All patients were staged according to the Tumor, Node and Metastasis (TNM) classification of the International Union against Cancer. The tumor invaded the submucosa (T1) in 30% of the cases (46/151). In 42% of the cases (63/151) it invaded the muscularis propria (T2) and in about 25% (38/151) it penetrated subserosal connective tissue (T3). In 4 patients (3%) the tumor infiltrated the visceral peritoneum or invaded adjacent structures (T4). Most gastric adenocarcinomas were either well differentiated (71/151, 47%) or moderately differentiated (70/151, 46%) with only 10 cases (10/151, 7%) poorly differentiated. Follow-up and survival data were available for all patients and were obtained from patient records.

Immunohistochemistry

Surgically resected samples were collected and tumor was confirmed by performing hematoxylin and eosin (H-E) staining on formalin fixed and paraffin embedded sections. Similarly, the presence of normal pathology in the adjacent normal tissues was also confirmed. Immunohistochemical labeling was done on 4μm tissue sections mounted on slides coated poly-L-lysine (Sigma, St. Louis, Missouri, USA) using the routine streptavidin-biotin-immunoperoxidase technique. Sections were deparaffinised in xylene, rehydrated through a series of graded alcohol to distilled water and microwaved in buffered sodium citrate. Endogenous peroxidase was blocked by incubating in hydrogen peroxidase with methanol followed by overnight incubation with monoclonal antibodies, anti-SMAD4 (clone B-8) and anti-SMAD7 (clone H-79), obtained from Santa-Cruz Biotechnology Inc., Santa-Cruz, California, USA. Novastatin Universal Detection kit (Ready to use, Novacastra Laboratories Ltd., Newcastle, UK) containing biotinylated secondary antibody was applied and staining was visualized using 3',3'-Diaminobenzidinetetrahydrochloride (Sigma Chemical Co., St. Louis, USA) solution as chromogen. The sections were counterstained in Mayer's haematoxylin, rinsed in water, and mounted in Di-N-Butyle Phthalate in Xylene. The brown product obtained was visualized and scored by light microscopy.

Immunohistochemical scoring was done independently by two senior pathologists and only samples with complete accordance in staining and histopathology were included in the study. The slides were scored as follows: 0 (no staining), 1+ (weak staining), 2+ (moderate staining), and 3+ (strong staining), a scoring system previously described by Hua et al. [17]. Paired adjacent normal tissue samples served as positive controls for each of the cases. There was complete accordance in all the cases.

Protein levels of both Smad4 and Smad7 were evaluated in adjacent normal gastric tissues and compared with those in the site of gastric adenocarcinoma. Immunoreactivity for both Smads was evaluated semiquantitively by the two pathologists and its expression was categorized as follows: "intact expression" (if ≥10% of the tumor cells were positive) and "loss of expression" (if <10% of the tumor cells were positive). Previous studies in which immunolabeling patterns have been correlated with Smad4 gene status have shown that both focal and diffusely positive labelings correlated with an intact Smad gene, whereas complete loss of labeling correlated with inactivation of Smad gene [18]. 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 [19]. For purposes of data analysis, both focal and diffusely positive lesions were considered to show intact Smad expression (pos-

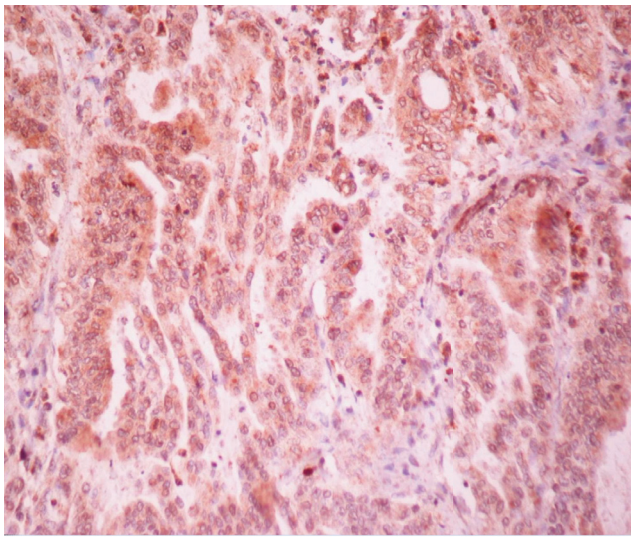

Figure 1. Positive expression of Smad4 in gastric carcinoma (Smad4 x 200).

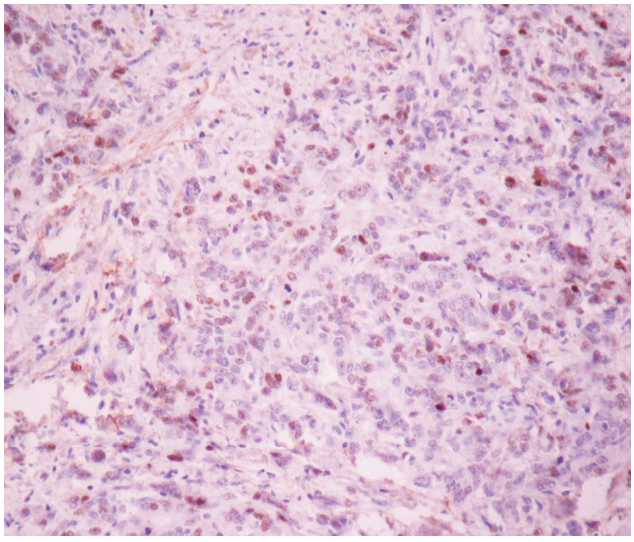

Figure 2. Nuclear staining of Smad4 in gastric carcinoma (Smad4 x 200).

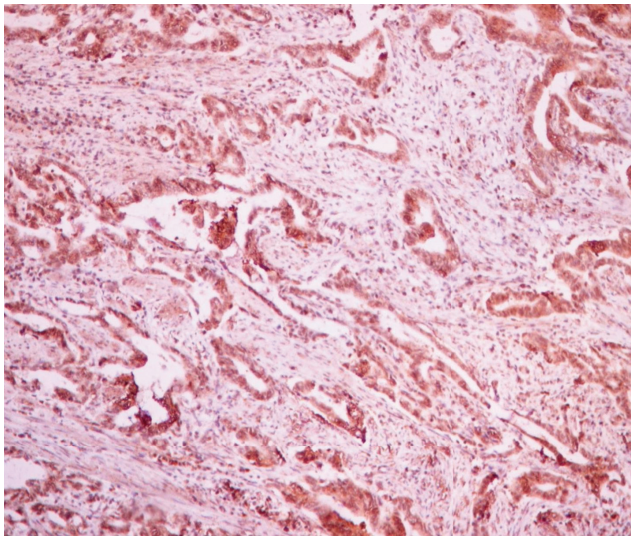

Figure 3. Positive expression of Smad7 in gastric carcinoma (Smad7 x 100).

itive), and complete loss of labeling was considered to show loss of Smad expression (negative).

Statistics

Fisher's exact test was used to compare Smad4 and Smad7 protein expression in normal and tumor tissue. 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 probability value of less than 0.05 was considered to be significant.

Results

Immunohistochemical staining for Smad4 and Smad7

Smad4 showed cytoplasmic as well as nuclear staining, which was both focal and diffuse (Figure 1 and 2). Most normal tissues showed strong to moderate Smad4 immunoreactivity (140/151, 93%), whereas in tumor tissues the rates of Smad4 expression were reduced. The difference in Smad4 protein levels in tumor tissue as compared to normal gastric tissue was highly significant on Fisher's exact test (two tailed p value = 0.0001).

Smad7 protein stained mainly in the cytoplasm of tumor cells, although occasional nuclear positivity was obtained in some normal gastric glands. Smad7 overexpression was observed in gastric cancer tissues (Figure 3) whereas no expression was observed in normal mucosa. The difference in the expression levels of Smad7 in normal gastric samples as compared to tumor samples was highly significant by Fisher's exact test (p = 0.0005).

Relationship between Smad4 or Smad7 expression and clinicopathologic findings

In our study Smad4 and Smad7 expression (low, moderate or strong) was observed in 86.7% (131/151) and 33.1% (50/151) of gastric adenocarcinoma tumor samples respectively. The correlation of Smad4 or Smad7 expression and the clinicopathologic findings are shown in Tables 1 and 2.

The rate of positive Smad4 expression was higher in female patients than in males (p=0.018) and in diffuse tumor type than in intestinal tumor type (p=0.014). The rates of positive Smad4 expression were also significantly higher in undifferentiated tumors than in well or moderately differentiated tumors. Smad4 protein expression

Table 1. Relationship of Smad4 protein expression with clinicopathologic findings in gastric cancer

	Cases (N=151)	*SMAD4 expression*		*p-value*
		Positive (N=131) N (%)	*Negative (N=20) N (%)*	
Sex				
Male	87	72 (80.1)	15 (17.2)	0.018
Female	64	59 (92.2)	5 (7.8)	
Age, years, mean ± SD		55.7±12.8	57.3±13.4	NS
Tumor depth (T)				0.017
T1	46	44 (95.7)	2 (4.3)	
T2	63	57 (90.5)	6 (9.5)	
T3	38	29 (76.3)	9 (23.7)	
T4	4	1 (25.0)	3 (75.0)	
Differentiation				0.017
Well	71	59 (83.1)	12 (16.9)	
Moderate	70	62 (88.5)	8 (11.5)	
Poor	10	10 (100.0)	0 (0)	
Lauren class				0.014
Intestinal	65	51 (78.4)	14 (21.6)	
Diffuse	86	80 (93.0)	6 (7.0)	
Venous invasion				NS
Absent	113	101 (89.3)	12 (10.7)	
Present	38	30 (78.9)	8 (21.1)	
Lymphatic invasion				NS
Absent	108	95 (87.9)	13 (12.1)	
Present	43	36 (83.7)	7 (16.3)	
Nodal metastasis				NS
Absent	84	75 (89.2)	9 (10.8)	
Present	67	56 (83.5)	11 (16.5)	

NS: non significant

was observed in 59 of 71 (83.1%) well differentiated gastric adenocarcinomas, in 62 of 70 (88.5%) moderately differentiated gastric adenocarcinomas and in all 10 (100%) samples of poorly differentiated gastric adenocarcinomas. However, the rate of Smad4-positive expression decreased as tumors invaded deeper layers (p=0.017). Smad4 expression was observed in 44 of 46 (89.1%) T1 tumors, in 57 of 63 (90.5%) T2 tumors, in 29 of 38 (76.3%) T3 tumors and in only 1 of 4 (25%) T4 tumors.

The rate of Smad7-positive expression was significantly higher in patients with differentiated tumors (p=0.006) than in those with undifferentiated and in intestinal type tumors (p=0.001) than in diffuse type tumors. Smad7 protein expression was obtained in 12 of 46 (26.1%) T1 tumors, in 21 of 63 (33.3%) T2 tumors, in 16 of 38 (42%) T3 tumors and in 1 of 4 (25%) T4 tumors. There was no significant correlation between Smad7 expression and sex, depth of invasion, vascular invasion, lymphatic invasion or lymph node metastasis.

Correlation between Smad4 or Smad7 expression and survival rate

The 5-year survival rate was 62% in patients with Smad4 positive tumors and 48% in patients with Smad4 negative tumors. The survival rate of patients with Smad4 positive expression was significantly higher than that of patients with negative expression in stage I (p=0.0042) and IV (p=0.005) tumors. No statistically significant difference was observed in stage II and III tumors. The survival rate of patients with tumors negative for Smad7 was significantly higher than that of patients with positive Smad7 expression in stage I (p=0.03) and stage III (p=0.05). No statistically significant difference was found in stage II and IV tumors (Table 3).

Table 2. Relationship of Smad7 protein expression with clinicopathologic findings in gastric cancer

	Cases (N=151)	*SMAD7 expression*		*p-value*
		Positive (N=50) N (%)	*Negative (N=101) N (%)*	
Sex				NS
Male	87	29 (33.3)	58 (66.7)	
Female	64	21 (32.8)	43 (67.2)	
Age, years, mean ± SD		64±3.45	66±2.5	NS
Tumor depth (T)				NS
T1	46	12 (26.0)	34 (74.0)	
T2	63	21 (33.3)	42 (66.7)	
T3	38	16 (42.1)	22 (57.9)	
T4	4	1 (25.0)	3 (75.0)	
Differentiation				0.002
Well	71	32 (45.0)	39 (55.0)	
Moderate	70	17 (24.2)	53 (75.8)	
Poor	10	1 (10.0)	9 (90.0)	
Lauren class				0.001
Intestinal	65	39 (60.0)	26 (40.0)	
Diffuse	86	11 (12.7)	75 (87.3)	
Venous invasion				NS
Absent	137	45 (32.8)	92 (67.2)	
Present	14	5 (35.7)	9 (64.3)	
Lymphatic invasion				NS
Absent	108	34 (31.4)	74 (68.6)	
Present	43	16 (37.2)	27 (62.8)	
Nodal metastases				NS
Absent	56	17 (30.3)	39 (69.7)	
Present	95	33 (34.7)	62 (65.3)	

NS: non significant

Table 3. Relationship between Smad4 and Smad7 expression and tumor stage

Stage	*Cases*	*SMAD4*			*SMAD7*		
	N	*Positive N (%)*	*Negative N (%)*	*p-value*	*Positive N (%)*	*Negative N (%)*	*p-value*
I	65	63 (96.9)	2 (3.1)	0.042	19 (29.2)	46 (70.8)	0.03
II	30	26 (86.7)	4 (13.3)	NS	12 (40.0)	18 (60.0)	NS
III	33	28 (84.5)	5 (15.5)	NS	10 (30.3)	23 (69.7)	0.05
IV	23	14 (60.8)	9 (39.2)	0.005	9 (39.2)	14 (60.8)	NS

NS: non significant

Discussion

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide. Epidemiological studies have identified many risk factors for gastric cancer, such *Helicobacter pylori (H. pylori)* infection, lower fiber intake and tobacco smoking [20]. However, only a fraction of individuals exposed to these factors develop gastric cancer during their lifetime, which suggests that genetic susceptibility plays an important role in gastric carcinogenesis.

Recent studies indicated that deregulated TGF-β family signaling has been implicated in

various human diseases, including autoimmune diseases, vascular disorders and cancer. TGF-β superfamily is composed of many multifunctional cytokines including TGF-βs, activins, inhibins and bone morphogenic proteins. These proteins regulate a variety of biological responses such as proliferation, differentiation, apoptosis and development. The canonical signaling pathway for the TGF-β family is through the Smad proteins which are pivotal intracellular mediators of TGF-β family members. 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. In the absence of ligand, the inhibitory Smads, Smad6 and Smad7, are localized predominantly in the nucleus. 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 [21].

In this study, we examined Smad4 and Smad7 expression in gastric carcinomas to elucidate their role in tumor progression.

In vitro and *in vivo* studies have shown that mutations in the Smad4 gene, located at 18q21 chromosome, play a significant role in Smad4 inactivation. The loss of Smad4 expression is a common feature of most human malignancies and is most prevalent in pancreatic and colorectal cancer [22]. Loss of heterozygosity studies have suggested that Smad4 is altered frequently in intestinal-type gastric carcinomas. In our study, Smad4 showed cytoplasmic as well as nuclear staining, which was both focal and diffuse. Most normal gastric mucosa cells showed strong to moderate immunoreactivity, whereas in tumor tissues the rates of Smad4 expression were reduced. We found that the loss of Smad4 protein expression was statistically significantly associated with intestinal type, as well as with male sex and well differentiated tumors. Our results also revealed that the loss of Smad4 expression was related to the depth of tumor invasion and poor survival. Our findings are in accordance with those described previously in the literature, even though there are studies which consider loss of Smad expression to be an independent prognostic factor for gastric adenocarcinoma. These different results may be attributable to patient selection and different cut-off positive values.

Smad7 inhibits TGF-β-induced transcriptional responses and has been reported to act as an important molecule for regulating TGF-β activity in human disease. In previous studies Smad7 was shown to be overexpressed in pancreatic cancer but little is known about whether Smad7 expression is associated with clinicopathological parameters such as tumor stage and prognosis. In our study, Smad7 protein stained mainly in the cytoplasm of tumor cells, wheras almost no expression was observed in normal gastric mucosa. Smad7 expression was statistically significantly more frequent in intestinal type and well differentiated gastric adenocarcinomas and significantly correlated with disease-free survival duration.

In subgroup analysis according to TNM stage, both Smad4 and Smad7 showed prognostic differences only in stage IB gastric cancer patients. This may prove to be very useful, as these patients may be candidates for adjuvant chemotherapy. Furthermore, our results showed that the expression patterns of Smad4 and Smad7 were inversely correlated with each other ($p<0.05$).

Finally, as far as 5-year overall survival in gastric cancer is concerned, Smad4 (+)/Smad7 (-) expression pattern was most favorable, while Smad4 (-)/Smad7 (+) expression pattern was most unfavorable ($p=0.001$).

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