Histol Histopathol (2005) 20: 543-549

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Smad4 expression in gastric adenoma and adenocarcinoma: Frequent loss of expression in diffuse type of gastric adenocarcinoma

J.Y. Kim[1], D.Y. Park[1], G.H. Kim[2], K.U. Choi[1], C.H. Lee[1], G.Y. Huh[1], M.Y. Sol[1], G.A. Song[2], T.Y. Jeon[3], D.H. Kim[3] and M.S. Sim[3]

[1]Department of Pathology, [2]Department of Internal Medicine and [3]Department of Surgery, College of Medicine, Pusan National University, Busan, Korea and Medical Research Institute, Pusan National University, Busan, Korea

Summary. Smads are signal transducers for the members of the TGF-ß superfamily. Of these Smads, Smad4 is essential for TGF-ß signaling. The purpose of this study was to elucidate Smad4 expression and localization in 65 gastric adenomas, 49 intestinal-type and 39 diffuse type of gastric adenocarcinomas (including 12 cases of fresh frozen tissue) using Real-time RT-PCR and immunohistochemistry. Real-time RT-PCR showed that intestinal type gastric adenocarcinomas have higher Smad4 mRNA expression than diffuse type gastric adenocarcinomas. Immunohistochemical stain for Smad4 revealed that expression of Smad4 was significantly lower in diffuse-type gastric adenocarcinoma than intestinal-type gastric adenocarcinomas. Also, higher Smad4 protein expression in intestinal type gastric adenocarcinomas than overall gastric adenoma was noted. The rate of reduced Smad4 expression was higher in advanced gastric cancer than early gastric cancer. These results suggest that Smad4 might play different roles in human gastric carcinogenesis, especially between intestinal type and diffuse type of gastric adenocarcinoma.

Key words: Gastric adenoma, Gastric adenocarcinoma, Smad4, Reat-time RT-PCR, Immunohistochemistry

Introduction

Smads, a small family of structurally related proteins, are signal transducers for the members of the transforming growth factor (TGF)-ß, superfamily (Massague, 1998). Smads are molecules of relative molecular mass 42K-60K with two regions of homology at the amino and carboxy terminals, termed Mad-homology domains, MH1 and MH2, respectively, which are connected with a proline-rich linker sequence (Heldin et al., 1997). Different members of the Smad family have different roles in signaling. Smad2 and Smad3 are activated via carboxy-terminal phosphorylation by type I TGF-ß receptor kinases and form heterotrimeric complexes with Smad4 and thereby act in a pathway-restricted fashion (Zhang et al., 1996). They are also called receptor-activated Smads. Smad4-receptor-activaor Smads complexes then translocate into the nucleus and act as TGF-ß induced transcriptional activators of target genes (Nakao et al., 1997). So, Smad4 acts as central mediator of TGF-ß functions (Zhang et al., 1997). 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 DPC4 (deleted in pancreatic carcinoma locus 4) (Hahn et al., 1996). Smad4 is also mutated in a significant proportion of colorectal cancers, and less frequently of breast, ovarian, head and neck, prostatic, esophageal and gastric cancers (Kim et al., 1996; Lei et al., 1996; Schutte et al., 1996; Takagi et al., 1996; MacGrogam et al., 1997). So it is suggested that Smad4 is often down regulated in cancer cells and this defect allows cancer cells to escape the growth inhibitory activities of TGF-ß.

Gastric cancer remains one of the most prevalent malignancies throughout the world (Stadtlander and Waterbor, 1999). More than 90% of gastric cancers are adenocarcinoma, which are divided into two histological types (intestinal and diffuse) by the Lauren classification (Lauren, 1965). Pathogenesis of the intestinal-type gastric adenocarcinoma has been connected to precursor changes such as chronic atrophic gastritis, intestinal metaplasia, and adenoma, whereas the diffuse type lacks well-recognized precursor lesions (Stadtlander and Waterbor, 1999; Schlemper et al., 2000). Furthermore, these two types of gastric adenocarcinoma seem to

Offprint requests to: Do Youn Park, M.D., PhD., Department of Pathology, College of Medicine, Pusan National University, Ami-1 Ga, Seo-Gu, Busan, Korea, 602-739. e-mail: pdy220@pusan.ac.kr

express distinct genetic backgrounds and carcinogenetic mechanisms are complex and poorly revealed (Stadtlander and Waterbor, 1999; Tahara et al., 1996). Also, little is known about patterns and localization of Smad4 expression in gastric adenoma and adenocarcinoma, especially related with mechanisms of gastric carcinogenesis. Therefore, by determining the Smad4 expression and localization in human gastric neoplasm, including adenoma and adenocarcinoma, we sought to determine the presumptive roles of Smad4 in the pathogenetic mechanisms of gastric carcinogenesis.

Materials and methods

Patients and specimens

A total of 88 gastric adenocarcinomas and 65 gastric adenoma specimens that underwent gastrectomy with lymph node dissection, and endoscopic mucosal resection, respectively, at Pusan National University Hospital between 1999 and 2001 were included in this study, from 103 male and 50 female patients with a mean age of 57.8 years (31-78 years). Formalin-fixed and paraffin embedded specimens were obtained from 153 patients. Fresh frozen tissue was available from 12 patients of gastric adenocarcinomas including matched gastric cancer tissues with surrounding normal tissues. The clinicopathological features of gastric adenocarcinoma were assessed in accordance with the General Rules for the Gastric Cancer Study in Japan (Japanese gastric cancer association, 1998). The histologic subtypes of gastric adenocarcinoma were subclassified as intestinal and diffuse type by the Lauren classification (Lauren, 1965). Epithelial dysplasia within adenoma was graded by two pathologists as either high-grade or low-grade dysplasia according to previously published criteria (Rugge et al., 2000).

Analysis of Smad4 mRNA expression by real-time RT-PCR in fresh frozen tissue

The fresh samples were received immediately after resection and divided into tumor and surrounding normal tissue, which were obtained from 12 patients of gastric adenocarcinomas. Frozen sections were prepared from matched tumor and surrounding normal tissue and examined to provide confirmation of the identification of tumor and surrounding normal tissue. After that, total RNA was isolated from fresh frozen tissue, using an RNeasy Kit (Qiagen, Santa Clarita, CA). cDNA was synthesized from 10ul of total RNA using 2ug of random hexamer (Pharmacia, Uppsala, Sweden), 10mM dNTP (Boehringer Mannheim, Mannheim, Germany) and 200 U of M-MLV reverse transcriptase (GIBCO BRL) in a final volume of 20 µl. The PCR primers and TaqMan probe for Smad4, 18S rRNA were designed using Beancon Desiner 3.0 (PREMIER Biosoft International, Palo Alto, CA, USA). The primer sequences for amplication were described as follows: for Smad4 (GeneBank NM_005359), sense primer 5'-TTGCCTCACCAAAACGG-3', antisense primer 5'-CACCAATACTCAGGAGCAGGATG-3', TaqMan probe 5'-FAM-CACCACCCGCCTATGCCGCCC-3'; for 18S rRNA (GeneBank M10098), sense primer 5'-CGTTGATTAAGTCCCTGCCCT-3', antisense primer 5'-TCAAGTTCGACCGTCTTCTCA-3', TaqMan probe 5'-FAM-ACACACCGCCCGTCGCTACCG-3'. The primers and probes were purchased from TIM MOLBIOL Syntheselabor (Berlin, Germay). Quantitative Real-time RT-PCR was performed using the Bio-Rad iCycler iQsystem (Bio-rad, Hercules, CA, USA). The following real-time PCR reaction mix was prepared, 12.5 µl of iQ Supermix (Bio-rad, Hercules, CA, USA), 2.5 µl 3 µM each primers, 2.5 µl 2 µM TaqMan probe, 3.0 µl cDNA. The PCR cycling conditions were 5 minutes at 95 °C followed by 45 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C (for 18S rRNA) and 58 ºC (for Smad4), and 30 seconds at 72 °C. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence generated by cleavages of the probe passes a fixed threshold above baselines. Relative Smad4 gene expression quantification for gastric adenocarcinoma tissue and matched surrounding normal tissue of each sample was calculated using the average Smad4 C_T value for each triplicate sample minus the average triplicate C_T value for the internal gene (18S rRNA), and differences between gastric adenocarcinoma tissue and matched surrounding normal tissue were calculated using the formula $2^{-(\Delta CT\ tumor - \Delta CT\ normal)}$ and expressed as a fold change in expression according to the comparative threshold cycle method ($2^{-\Delta\Delta C}T$) (Livak and Schmittgen, 2001). Mann-Whitney U tests were used to compare fold changes between intestinal and diffuse type gastric adenocarcinoma. Significance was defined as $p < 0.05$.

Analysis of Smad4 protein expression and localization by immunohistochemical staining

Sections were dewaxed and rehydrated according to a standard procedure, and washed with PBS. For immunohistochemcial stain of Smad4, sections were heated in a microwave oven at 600W for 2x5minutes in 0.01M citrate buffer, pH=6.0. Sections were immersed in 3% H_20_2 to quench endogenous peroxidase activity, and unspecified binding was blocked in 5% normal rabbit serum (0.1% BSA in PBS). Immunohistochemical stain was performed by the avidin-biotin peroxidase complex method with aminoethylcarbazole as a chromogen using the Vetastain ABC elite kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin solution. To detect Smad4, goat polyclonal antibodies against Smad4 (B8, Santa-Cruz, CA, USA) was used at a dilution of 1:50. In the negative control group, 5% normal rabbit serum was used in place of the primary antibody.

The expression of Smad4 protein was analyzed as described previously (Xiangming et al., 2001). The expression of Smad4 was compared with that of adjacent gastric glandular cells located away from the tumor. Tumor cells that stained as strongly as adjacent gastric glandular cells were considered positive (+), whereas those that stained weaker than adjacent gastric glandular cells or did not stain at all were considered weak (+/-) or negative (-), respectively. Tumors were then classified according to their expression of Smad4 upon overview of the section, being considered to have "Smad4 preserved expression" if >50% of the tumor cells were positive. Tumors classified as having "reduced expression" were those that did no fit into the above categories. Statistical analysis between preserved group and reduced group of Smad4 expression was performed by the chi-square test and Fischer probability exact test. Significance was defined as $p<0.05$.

Results

Expression of Smad4 mRNA in fresh frozen tissue

Of the gastric cancer available fresh frozen tissue, 5 cases were diffuse type of gastric adenocarcinoma and 7 cases were intestinal type of gastric adenocarcinoma.

Table 1. Expression of Smad4 mRNA in intestinal type and diffuse type of gastric adenocarcinoma.

	ΔC_T OF TUMOR	ΔC_T OF NORMAL SURROUNDING TISSUE	FOLD CHANGE* ($2^{-\Delta\Delta CT}$)	MEAN ± STANDARD DEVIATION
Intestinal	8.69	13.90	37.01	
	13.40	18.90	45.25	
	7.90	10.60	6.50	
	7.40	9.90	5.66	19.02±15.80
	6.00	10.00	16.00	
	7.40	11.30	14.93	
	10.80	13.77	7.84	
Diffuse	5.30	5.80	1.23	
	3.50	3.70	1.15	0.70±0.51
	12.20	11.78	0.75	
	10.70	7.90	0.14	
	13.20	11.10	0.23	

*: fold changes between intestinal and diffuse, p=0.004.

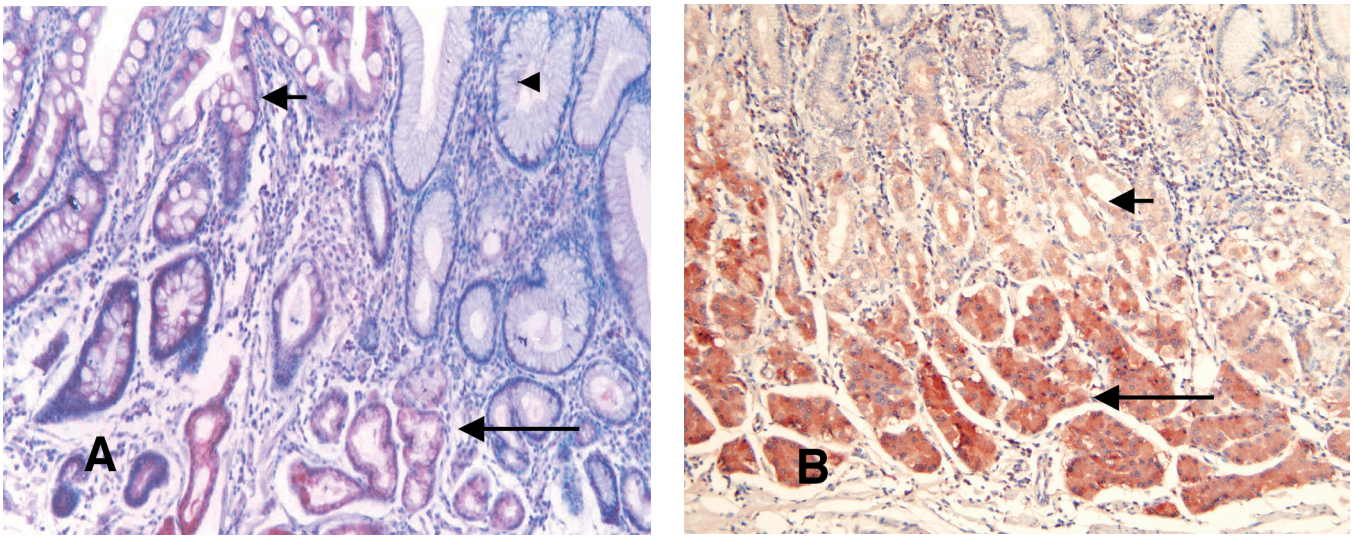

Fig. 1. Immunohistochemical stain for Smad4 protein. Normal gastric antral mucosa adjacent to tumor shows strong cytoplasmic Smad4 positivity in gastric mucous cells (larger arrow) and intestinal metaplastic foveolar epithelial cells (smaller arrow), while weak staining in foveolar epithelial cells (arrowhead) **(A)**. Normal gastric body mucosa adjacent to tumor shows more strong cytoplasmic Smad4 expression in the base of the oxyntic glands (larger arrow) than neck and isthmus (smaller arrow) of the oxyntic glands **(B)**. x 100

Intestinal type of gastric adenocarcinoma showed higher fold changes of Smad4 mRNA expression compared to surrounding normal tissue than diffuse type of gastric adenocarcinomas (Table 1).

Localization of Smad4 protein normal gastric mucosa, gastric adenoma and adenocarcinoma

Normal gastric mucosa adjacent to adenoma or adenocarcinoma showed strong Smad4 positivity in the glandular compartment of gastric mucosa, but weak staining in the foveolar compartment of gastric mucosa. Gastric antral/pyloric and cardiac mucosa adjacent to tumor showed strong cytoplasmic Smad4 positivity in gastric mucous cells and intestinal metaplastic foveolar epithelial cells, while weak staining in foveolar epithelial cells. Gastric body mucosa adjacent to tumor showed stronger cytoplasmic Smad4 expression in the base of the oxyntic glands than neck and isthmus of the oxyntic glands (Fig. 1) Gastric adenoma and adenocarcinoma showed diffuse cytoplasmic staining with occasional nuclear positivity of Smad4 (Fig. 2).

Smad4 protein expression in gastric adenoma and adenocarcinoma

Of the 65 cases of gastric adenoma, the rates of preserved and reduced Smad4 expression were 61.5% and 38.5%, respectively. Expression of Smad4 protein in gastric adenoma was not statistically associated with adenoma size, or location. Expression of Smad4 protein showed a more preserved pattern in high grade adenoma (11 cases/14 cases, 78.5%) than low grade adenoma (29

Table 2. Expression of Smad4 protein in gastric adenoma in regard to dysplasia.

	Expression of Smad4 protein		
	Case No.	Preserved	Reduced
Gastric adenoma*	65	40	25
Low grade	51	29	22
High grade	14	11	3

*: p=0.216.

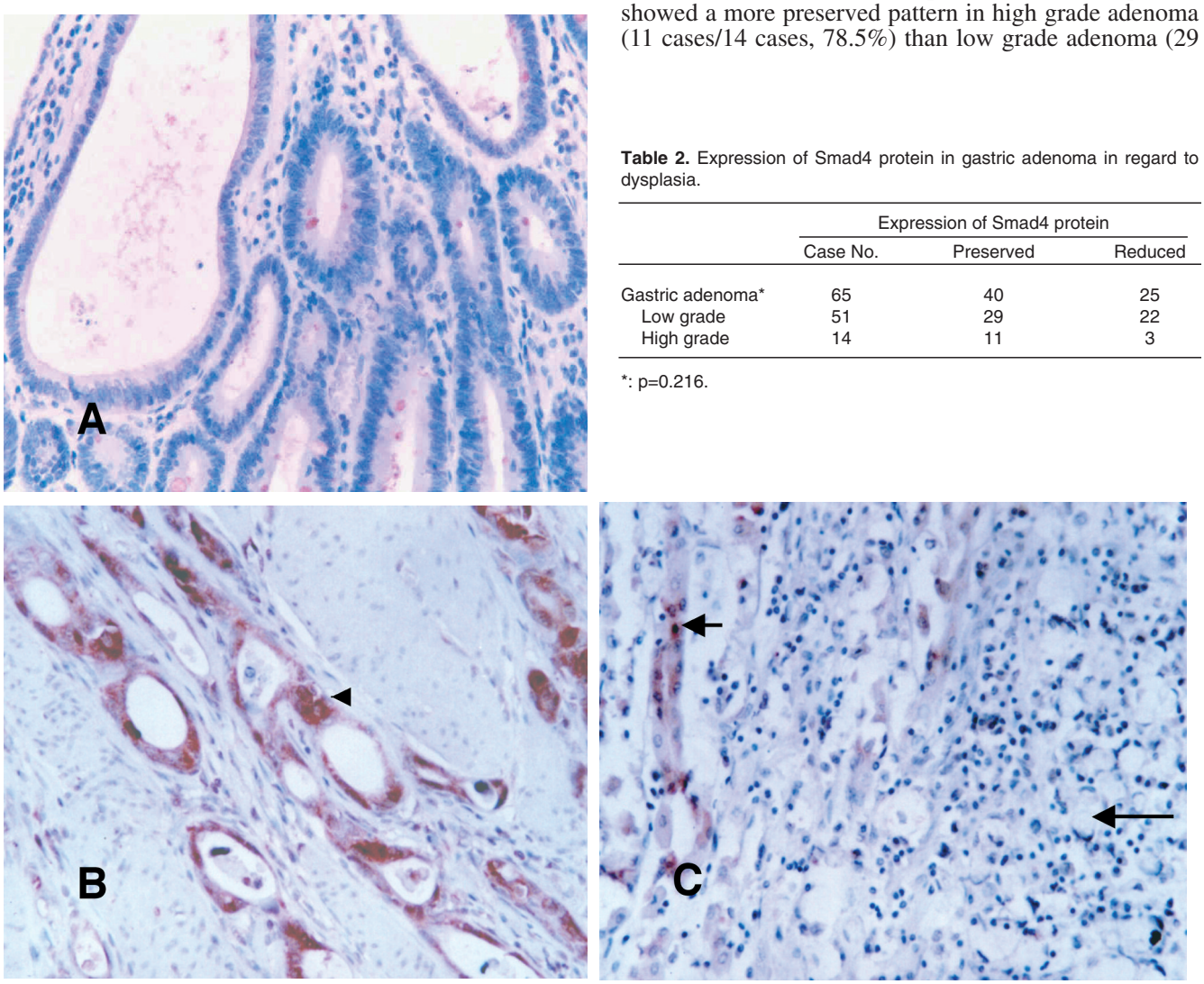

Fig. 2. Immunohistochemical stain for Smad4 protein. Intestinal type gastric adenocarcinoma **(B)** showed increased Smad4 positivity than gastric adenoma **(A)**. Notice cytoplasmic and nuclear Smad4 positivity (arrowhead) are seen in intestinal type gastric adenocarcinoma. Diffuse type gastric adenocarcinoma (signet ring cells) are negative for Smad4 (larger arrow) **(C)**. Notice smad4 positively stained gastric glandular cells (small arrow). x 200

cases/51 cases, 56.9%), but there was no statistical significance between them (Table 2) (Fig. 2A). Higher Smad4 protein expression in intestinal type gastric adenocarcinomas than overall gastric adenoma was noted (Table 3).

Of the 88 cases of gastric adenocarcinoma, the rates of preserved and reduced Smad4 expression were 68.1% and 31.9%, respectively (Table 4). Expression of Smad4 protein was significantly correlated with histologic types of gastric adenocarcinoma. That is, intestinal type of gastric adenocarcinoma showed more preserved Smad4 expression than diffuse type of gastric adenocarcinoma (Fig. 2B,C). Also, the rate of Smad4 reduced expression was higher in serosa and subserosa infiltrative advanced gastric cancer (15 cases/34 cases, 44.1%) than early gastric cancer and muscularis infiltrative advanced gastric cancer (17 cases/54 cases, 31.5%). But expression of Smad4 was not related to other clinicopathological factors, including lymph node metastatsis, lymphovascular tumor emboli, tumor size and tumor stage.

Discussion

In the present study, immunostain for Smad4 showed both cytoplasmic and some nuclear positivity in accordance with other reports of esophageal (Fukuchi et al., 2002), gastric (Xiangming et al., 2001) and colorectal cancer (Korchynskyi et al., 1999). Normal gastric mucosa adjacent to adenoma or adenocarcinoma showed strong Smad4 positivity in glandular compartment of gastric mucosa, while weak staining in the foveolar compartment of gastric mucosa. We revealed that the intestinal type of gastric adenocarcinoma showed more preserved Smad4 expression than gastric adenoma in our study. Because development of intestinal-type gastric adenocarcinoma has been highly associated with adenoma, study of Smad4 expression in gastric adenoma and adenocarcinoma might contribute to unravel the adenoma-carcinoma sequences in the stomach. Generally, Smad4 acts as a central mediator of TGF-ß signaling and, simultaneously, as tumor suppressor gene. Therefore, loss of Smad4 expression is responsible, at least in part, for the resistance of tumor cells to the anti-proliferative effects of TGF-ß during the adenoma-carcinoma sequences. This contention is in agreement with studies which found that noninvasive tumors showed more preserved Smad4 expression than invasive tumors in pancreatic neoplasms (Iacobuzio-Donahue et al., 2000; McCarthy et al., 2001). Also, in animal studies, haploid loss of Smad4 showed gastric polyposis and cancer in mice (Xu et al., 2000). But, in our study, intestinal type of gastric adenocarcinoma showed more preserved Smad4 expression than overall adenoma. These observations might suggest at least two possible explanations. The first is that TGF-ß signaling through Smad4 might have no significant roles in gastric carcinogenesis, especially development of intestinal type adenocarcinoma. This contention is partly in agreement with previous reports which showed that loss of Smad4 function which is revealed as Smad4 mutations is infrequent in gastric adenocarcinoma and target for loss on chromosome 18q in gastric adenocarcinoma is not Smad4 (Lei et al., 1996; Powell et al., 1997). Also, previous report (Tsukashita et al., 2001) revealed that high-grade adenoma and intramucosal carcinoma of stomach showed gastric mucin phenotype, compared to higher intestinal mucin phenotype in low-grade gastric adenoma. They suggested that adenoma-carcinoma sequence is not a major pathway of gastric carcinogenesis, but instead that gastric adenocarcinomas arise de novo. The second is that TGF-ß might play complex roles in gastric carciongenesis by behaving as tumor suppressor on the early stages, such as gastric adenoma, but as tumor promoter (through angiogenesis

Table 3. Expression of Smad4 protein in gastric adenoma and intestinal type gastricadenocarcinoma.

	Expression of Smad4 protein		
	Case No.	Preserved	Reduced
Gastric adenoma	65	40	25
Intestinal type adenocarcinoma	49	44	5

p=0.001.

Table 4. Relationship of Smad4 protein expression to clinicopathologic features in 88 cases of gastric adenocarcinoma.

	Case No.	Smad4 expression		p value
		Preserved	Reduced	
Age(years)				
≤ 50	30	18	12	NS
> 50	58	42	16	
Tumor size(cm)				
≤ 2.5	45	30	15	NS
> 2.5	43	30	13	
Invasion Depth				
Mucosa	28	18	10	0.049
Submucosa	16	14	2	
Muscularis	10	5	5	
Subserosa+SE+SI	34	19	15	
Lauren classification				
Intestinal	49	44	5	0.000
Diffuse	39	16	23	
Lymph node metastasis				
Negative	51	34	17	NS
Positive	37	26	11	
Lymphovascular tumor Emboli				
Negative	57	40	17	NS
Positive	31	20	11	

SE: serosa exposed; SI: serosa infiltrated.

or antitumor immune responses) at later stages, such as gastric adenocarcinoma. Smad4 may be involved in both roles in carcinogenesis (Reiss, 1996; Akhurst and Balmain, 1999). This contention is partly in agreement with our study which showed a decrease of Smad4 in gastric adenoma and an increase of Smad4 in intestinal type of gastric adenocarcinoma. In the current paradigm, TGF-ß has suppressor activities dominantly in normal tissue, but during carcinogenesis, TGF-ß expression and cellular responses tip the balance in favor of its carcinogenetic activities (Wakefield and Roberts, 2002). Taken together, it is suggested that Smad4 might have some roles in favoring progression of gastric adenoma to intestinal type adenocarcinoma. But more extensive studies are needed to reveal exact roles of Smad4 in gastric adenoma and intestinal type of gastric adenocarcinoma.

Perhaps one of the most interesting findings of this study is that diffuse type of gastric adenocarcinoma showed frequent loss of Smad4 expression. Gastric cancer behaves as two distinct diseases. This was first recognized by Lauren, who distinguished an intestinal type of gastric adenocarcinoma from a diffuse type of gastric adenocarcinoma based on its histologic characteristics (Lauren, 1965). Little is known about the exact carcinogenetic mechanisms of gastric adenocarcinoma. Many gastric adenocarcinomas arise on a background of chronic atrophic gastritis with intestinal metaplasia. Generally, intestinal type of gastric adenocarcinoma, which occur more frequently in elderly men, are associated with better survival. Also, many differences in the molecular alternations between the intestinal type and diffuse type of gastric adenocarcinoma are seen (Ming, 1998). In our study, diffuse type of gastric adenocarcinoma shows more frequent loss of Smad4 expression than intestinal type of gastric adenocarcinoma. These results suggested that different roles of Smad4 in TGF-ß signaling pathway in human gastric carcinogenesis in regard to the Lauren histologic classification. But, a previous report (Xianming et al, 2001) revealed that there was no statistically significant difference of Smad4 expression between intestinal and diffuse stomach cancer. Although, there is a difference in sample selection, that is, Xianming et al studied only in advanced gastric cancer, we cannot suggest exact causes of these differences. Because TGF-ß may play a pivotal role in the anti-mitogenic and anti-invasive activities of the cells, effectively functioning as tumor suppressor (Prunier et al., 1999; de Caestecker et al., 2000), preserved Smad4 expression in intestinal type of gastric adenocarcinoma suggests that intestinal type of gastric adenocarcinoma might have better survival rate than diffuse type of gastric adenocarcinoma. But, we cannot suggest the exact mechanisms of loss of Smad4 expression in diffuse type of gastric adenocarcinoma in our study. Smad4 mutations is infrequent in gastric adenocarcinoma (Lei et al., 1996; Powell et al., 1997). So mechanisms of Smad4 down-regulation are uncertain and some other mechanisms of down-regulation of Smad4 other than Smad4 mutation, especially in diffuse type of gastric adenocarcinomaa, might be present, such as posttranscriptional modifications, methylation abnormalities. Therefore, more in vitro and in vivo studies are needed to confirm exact mechanisms of Smad4 down regulation in diffuse type of gastric adenocarcinoma.

In our study, the rate of Smad4 reduced expression was increased in accordance with depth of invasion. These results suggest that down regulation of Smad4 expression might be associated with increased invasiveness and aggressiveness in gastric adenocarcinoma. This contention is in agreement with studies, which show that preserved Smad4 expression is a favorable prognostic factor in patients with advanced gastric cancer and squamous cell carcinoma of esophagus (Xiangming et al., 2001; Fukuchi et al., 2002). But, TGF-ß might play complex roles in carcinogenesis by behaving as tumor suppressor or tumor promoter (Reiss, 1996; Akhurst and Balmain, 1999). Therefore, more in vitro and in vivo studies are needed to confirm exact roles of Smad4 in gastric carcinogenesis.

Taken together, it is suggested that diffuse type of gastric adenocarcinoma has more frequent loss of Smad4 expression and this mechanism might explain some different biologic characteristics between diffuse and intestinal type of gastric adencarcinoma in regard to TGF-ß functional activities.

Acknowledgements. This study was supported by Medical Research Institute Grant (2002-01-38, 2003-05), Pusan National University Hospital.

References

Akhurst R.J. and Balmain A. (1999). Genetic events and the role of TGF beta in epithelial tumour progression. J. Pathol. 187, 82-90.

de Caestecker M.P., Piek E. and Roberts A.B. (2000). The role of TGF-beta signaling in cancer. J. Natl. Cancer Inst. 92, 1388-1402.

Fukuchi M., Masuda N., Miyazaki T., Nakajima M., Osawa H., Kato H. and Kuwano H. (2002). Decreased Smad4 expression in the transforming growth factor-beta signaling pathway during progression of esophageal squamous cell carcinoma. Cancer 95, 737-743.

Hahn S.A., Schutte M., Hoque A.T., Moskaluk C.A., da Costa L.T., Rozenblum E., Weinstein C.L., Fischer A., Yeo C.J, Hurban R.H. and Kern S.E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271, 350-353.

Heldin C.H., Miyazone K. and ten-Dijke P. (1997). TGF-ß, signaling from cell membrane to nucleus through Smad poteins. Nature 390, 465-471.

Iacobuzio-Donahue C.A., Wilentz R.E., Argani P., Yeo C.J., Cameron J.L., Kern S.E. and Hruban R.H. (2000). Dpc4 protein in mucinous cystic neoplasms of the pancreas: frequent loss of expression in invasive carcinomas suggests a role in genetic progression. Am. J. Surg. Pathol. 24, 1544-1548.

Japanese gastric cancer association. (1998). Japanese classification of gastric carcinoma. Gastric Cancer 1, 10-24.

Kim S.K., Fan Y., Papadimitrakopoulou V., Clayman G., Hittelman W.N., Hong W.K., Lotan R. and Mao L. (1996). DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. Cancer Res. 56, 2519-2521.

Korchynskyi O., Landstrom M., Stoika R., Funa K., Heldin C.H., ten Dijke P., and Souchelnytskyi S. (1999) Expression of Smad proteins in human colorectal cancer. Int. J. Cancer 82, 197-202.

Lauren T. (1965). The two histologic main types of gastric carcinomas: an attempt at a histoloclinical classification. Acta. Pathol. Microbiol. Scand. 64, 31-49.

Lei J., Zou T.T., Shi Y.Q., Zhou X., Smolinski K.N., Yin J., Souza R.F., Appel R., Wang S., Cymes K., Chan O., Abraham J.M., Harpaz N. and Melzer S.J. (1996). Infrequent DPC4 gene mutation in esophageal cancer, gastric cancer and ulcerative colitis-associated neoplasms. Oncogene 13, 2459-2462.

Livak K.J. and Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method. Methods 25, 402-408.

MacGrogam D., Pegram M., Slamon D. and Bookstein R. (1997). Comparative mutations analysis of DPC4 (Smad4) in prostatic and colorectal carcinomas. Oncogene 15, 1111-1114.

Massague J. (1998). TGF-ß, signal transduction. Annu. Rev. Biochem. 67, 753-791.

McCarthy D.M., Brat D.J., Wilentz R.E., Yeo C.J., Cameron J.L., Kern S.E. and Hurban R.H. (2001). Pancreatic intraepithelial neoplasia and infiltrating adenocarcinoma: analysis of progression and recurrence by DPC4 immunohistochemical labeling. Hum. Pathol. 32, 638-642.

Ming S.C. (1998). Cellular and molecular pathology of gastric carcinoma and precursor lesions: A critical review. Gastric Cancer 1, 31-50

Nakao A., Imamura T., Souchelnytskyi S., Kawabata M., Ishisaki A., Oeda E., Tamaki K., Hanai J., Heldin C.H., Miyazono K. and ten Dijke P. (1997). TGF-ß, receptor-mediate signalling through Smad2, Smad3 and Smad4. EMBO J. 16, 5353-5362.

Powell S.M., Harper J.C., Hamilton S.R., Robinson C.R. and Cummings O.W. (1997). Inactivation of Smad4 in gastric carcinomas. Cancer Res. 57, 4221-4224.

Prunier C., Mazars A., Noe V., Bruyneel E., Mareel M., Gespach C. and Atfi A. (1999). Evidence that Smad2 is a tumor suppressor implicated in the control of cellular invasion. J. Biol. Chem. 274, 22919-22922.

Reiss M. (1999). TGF-beta and cancer. Microbes Infect. 1, 1327-1347

Rugge M., Correa P., Dixon M.F., Hattori T., Leandro G., Lewin K., Riddell R.H., Sipponen P. and Watanabe H. (2000). Gastric dysplasia: the Padova international classification. Am. J. Surg. Pathol. 24, 167-176.

Schlemper R.J., Riddell R.H., Kato Y., Brochard F., Cooper H.S., Dawsey S.M., Dixon M.F., Fenoglio-Preiser C.M., Flejou J.F., Geboes K., Hattori T., Hirota T., Ltabashi M., Iwafuchi M., Iwashita A., Kim Y.I., Krichner T., Klimpfunger M., Koike M., Lauwers G.Y., Lewin K.J., Oberhuber G., Offner F., Price A.B., Rubio C.A., Shimizu M., Shimoda T., Shipponen P., Solcia E., Stolte M., Watanabe H. and Yamabe H. (2000). The Vienna clssification of gastrointestinal epithelial neoplasia. Gut 46, 251-255.

Schutte M., Hruban R.H., Hedrick L., Cho K.R., Nadasdy G.M., Weinstein C.L., Bova G.S., Saacs W.B., Cairns P., Nawroz H., Sidransky D., Casero R.A. Jr, Meltzer P.S., Hahn S.A. and Kern S.E. (1996). DPC4 gene in various tumor types. Cancer Res. 56, 2527-2530.

Stadtlander C.T. and Waterbor J.W. (1999). Molecular epidemiology, pathogenesis and prevention of gastric cancer. Carciongenesis 20, 2195-2208.

Tahara E., Semba S. and Tahara H. (1996). Molecular biological observations on gastric cancer. Semin. Oncol. 23, 307-315.

Takagi Y., Kohmura H., Futamura M., Kida H., Tanemura H., Shimokawa K. and Saji S. (1996). Somatic alternation in the DPC4 gene in human colorectal cancers in vivo. Gasteroenterology 111, 1369-1372.

Tsukashita S., Kushima R., Bamba M., Sugihara H. and Hattori T. (2001). MUC gene expression and histogenesis of adenocarcinoma of the stomach. Int. J. Cancer 94, 166-170.

Wakefield L.M. and Roberts A.B. (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. Curr. Opin. Genet. Dev. 12, 22-29.

Xiangming C., Natsugoe S., Takao S., Hokita S., Ishigami S., Tanabe G., Baba N., Kuroshima K. and Aikou T. (2001). Preserved Smad4 expression in the transforming growth factor signaling pathway is a favorable prognostic factor in patients with advanced gastric cancer. Clin. Cancer Res. 7, 277-282.

Xu X., Brodie S.G., Yang X., Im Y.H., Parks W.T., Chen L., Zhou Y.X., Weinstein M., Kim S.J. and Deng C.X. (2000). Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. Oncogene 19, 1868-1874.

Zhang Y., Feng X.H., Wu R.Y. and Derynck R. (1996). Receptor-associated Mad homologues synergize as effectors of the TGF-ß, response. Nature 383, 168-172

Zhang Y., Musci T. and Derynck R. (1997). The tumor suppressor Smad4/DPC4 as a central mediator of Smad function. Curr. Biol. 7, 270-276.

Accepted January 10, 2005