Immunohistochemical Expression of P15 (INK4B) and SMAD4 in Advanced Gastric Cancer

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Abstract. *Background: P15 (a cyclin- D- kinase inhibitor) and SMAD4 (a signal transducer of the TGF-β pathway) are two closely related proteins which may have an important role in gastric carcinogenesis. Materials and Methods: Sixty-three gastric carcinomas were studied. P15 and SMAD4 immunohistochemical expression was assessed with the DCS114.1 and BC/B8 mouse monoclonal antibodies, respectively. The results were tested for correlation with several clinicopathological parameters and patient survival. Results: Fifteen out of the 18 (83%) P15-positive tumors also exhibited preserved SMAD4 positivity (p=0.001). Loss of P15 expression was more frequent in the intestinal type of carcinoma (p=0.047). The survival rates were significantly higher for patients with reduced SMAD4 expression, in cases of well- or moderately differentiated tumors (p=0.042). Conclusion: P15 gene silencing might be an important event in the tumorigenesis of gastric adenocarcinomas of the intestinal type. Smad4 may behave as a tumor promoter in low grade tumors. The observed association between P15 and SMAD4 expression supports the inductive role of Smad4 in p15 transcription.*

During the past two decades there has been a notable steady decline in the incidence of gastric cancer in the western world. Despite this, carcinoma of the stomach remains one of the most frequent causes of death from malignancies worldwide (1, 2). Tumorigenesis in the stomach is thought to be a long-term, multistep process resulting from the accumulation of various environmental and genetic factors (3). Infection with *Helicobacter pylori* (4) or Epstein Barr virus (5), and dietary aspects are strongly implicated in the initiation of gastric cancer. Recent reports have pointed out that there might be a contribution of inherited genetic factors to sporadic gastric cancer but epigenetic changes seem to play the most important role (3).

Cyclin-dependent kinase inhibitor p15, a candidate tumor suppressor gene, has been frequently reported to be inactivated in a large number of malignancies (6-11). P15 gene inactivation may be a consequence of loss of heterozygocity (LOH), homozygous deletion, point mutation and promoter hypermethylation. In gastric carcinogenesis, mutations or LOH of *p15* have rarely been identified (12, 13), while the aberrant methylation of the promoter region of the gene has been proved to be the most important mechanism of inactivation (11). Recent studies have shown that *p15* promoter hypermethylation was also detected in precancerous lesions of the stomach, such as intestinal metaplasia, implying that this mechanism might play an important role in the early steps of gastric carcinogenesis (14, 15).

Smad4 (DPC4), a gene that is frequently deleted or mutated in pancreatic carcinoma (16), has also attracted the interest of many investigators for its potential role in gastric carcinogenesis. This was mainly based on the fact that LOH studies of gastric carcinomas revealed the frequent allelic loss on the chromosomal 18q arm (17), where the DPC4/SMAD4 gene is located. In addition, observations on the genetic background of juvenile polyposis (JP) syndrome support the idea that *Smad4* might play a role as a tumor suppressor gene in gastric cancer (18).

Since *Smad4* seems to be involved in *p15* transcription (19), in the present study the expression patterns of p15 and Smad4 proteins in human gastric cancer was investigated by immunohistochemistry and correlated with the survival rate of the patients as well as with other clinicopathological parameters.

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Materials and Methods

From 1998 to 2003, 63 gastric cancer cases were retrieved from the archives of the Pathology departments of two affiliated hospitals. All the samples were obtained with the consent of the patient or the bereaved and after the approval of the local Ethics Committee. There were 4 (6.35%) stage I (IB), 10 (15.87%) stage II, 42 (66.67%) stage III (26IIIA and 16 IIIB) and 7 (11.11%) stage IV tumors. The patient' follow-up time lasted from 50-80 months, or until their death. The overall 5-year survival rate was 24.5%.

The histological evaluation was based on the Lauren classification system (20). There were 33 (52.38%) cases of intestinal, 18 diffuse (28.57%) and 12 (19.05%) mixed carcinomas. Regarding the degree of differentiation, 10 cases (15.87%) were characterized as well-differentiated, 14 (22.22%) as moderately and 39 (61.90%) as poorly differentiated. For statistical purposes, all of the cases of the diffuse type and most of the mixed ones were conventionally included in the poorly differentiated tumor group.

Thirty-two tumors (50.79%) were located at the distal part of the stomach (pyloric area), 15 (23.81%) at the proximal part (fundus and corpus), 7 (11.11%) invaded the gastroesophageal junction, 5 (7.94%) occupied the entire stomach (linitis plastica) and 4 (6.35%) were found on the gastric remnant from a previous Billroth II gastrectomy for benign ulcer, 17-23 years earlier.

Immunohistochemical staining. Immunohistochemical staining against P15 and SMAD4 was performed by the EnVision+ System-HRP. One formalin-fixed paraffin-embedded block was selected from each tumor and cut into 3-μm-thick sections. The tissue sections were deparaffinized, rehydrated and treated with a hydrogen peroxide solution for 30 min to quench endogenous peroxidase. The slides were then immersed in Trilogy solution (EDTA and emulsifiers-Cell Marque, Rocklin, CA, USA) and steamed at 80˚C for 1 hour. After cooling for 20 min, they were incubated with the primary antibody, mouse monoclonal antibody to P15 (1:100 dilution, clone DCS114.1; GeneTex, Inc., San Antonio, TX, USA) and mouse monoclonal antibody to SMAD4 (1:75 dilution, clone BC/B8; Biocare Medical, Concord, CA, USA) for 1 hour at room temperature and then incubated for 45 min with the anti-mouse HRP-labelled polymer (EnVision+ System-HRP; DakoCytomation, Glostrup, Denmark). Finally, the sections were treated with a diaminobenzidine (DAB) chromogenic substrate (BioGenex, San Ramon, CA, USA) for 10 min, counterstained with hematoxylin, dehydrated and coverslipped.

Some slides were treated as negative controls by omitting the primary antibody. Strong staining of normal gastric epithelium served as the positive internal control for SMAD4. Sections from a case with known strong positivity served as the positive control for P15.

Immunohistochemical scoring. The sections were examined using light microscopy by two independent observers (K.P and S.S) who were unaware of the clinicopathological data. Interobserver variation was resolved by simultaneous dual re-evaluation. The staining was evaluated over the entire tumor section. The immunoreactivity for P15 was evaluated with reference to both the staining intensity and the positively stained area. The staining intensity was scored as follows: 0, none; 1, weak; 2, moderate and 3, strong. The positively stained area was expressed as the percentage of the whole cancer area and scored as: 0, none; 1: 0-25%, 2: 26-50% and 3: >51%. Slides of tumors where the sum of scores was less than or equal to 2 (≤2) were defined as exhibiting negative expression and those with scores more than or equal to 3 (≥3) as exhibiting positive expression. Smad4 expression was evaluated using the scoring system proposed by Xiangming *et al.* (21). In brief, the expression of SMAD4 in tumor cells was compared with that of normal epithelial cells located away from the tumor. Tumor cells were considered as positive when they stained as strongly as normal epithelial cells. Weaker staining intensity or no staining was considered as weak and negative respectively. Tumors were considered as having SMAD4 preserved expression if >50% of the tumor cells were positive. All other tumors were considered as having SMAD4 reduced expression.

Statistical analysis. Differences in percentages between two groups were assessed using the Fisher exact test, while continuous variables were compared using the Mann-Whitney test. The patient survival was defined as the time from the initial diagnosis to the patient's death or last follow-up. The time to event distributions were estimated by the Kaplan-Meier method and comparisons were performed using the log-rank test. In order to explore the effect of patient and tumor characteristics on the survival time, univariate Cox regression analysis was performed. A multivariate Cox model was used, including patient gender (male *vs.* female), age, degree of tumor differentiation (poor *vs.* good or moderate), nodal stage (0, 1, 2), metastatic status (1 *vs.* 0), P15 and SMAD4 expression (positive/negative). The subclass of significant variables was identified through the backwards selection procedure. For all the statistical tests $p<0.05$ was regarded as significant. Analysis was performed using SPSS 10 (SPSS Inc. Chicago, IL, USA).

Results

P15 immunostaining was nuclear (Figure 1) with some cases featuring both nuclear and weak cytoplasmic reaction. Staining was heterogeneous with no differences between the superficial and the invasive part of the tumor. The proportion of cases that were considered as positive for P15 was 28.57% (18 cases).

SMAD4-positive expression was observed mainly in the cytoplasm and on occasions simultaneously in the nucleus (Figure 2). Normal epithelial cells showed homogeneous staining for SMAD4. In the cancer tissue, SMAD4 expression was preserved in 47.6% (30 cases) and reduced in 52.4% (33 cases). In accordance with the observation of previous investigators (21), as a rule, SMAD4 expression was better preserved in the superficial layers of the tumor and was lower in the deeper layers within the same tissue section.

In our study, 15 of the 18 (83%) P15-positive tumors also exhibited preserved SMAD4 positivity, indicating an association between *p15* and *Smad4* protein expression ($p=0.001$). The relation of *p15* and *Smad4* protein expression to the clinicopathological data under investigation is shown in Table I.

After a median follow-up of 72 months [95% confidence interval (CI) 44-100 months], 53 deaths had been recorded.

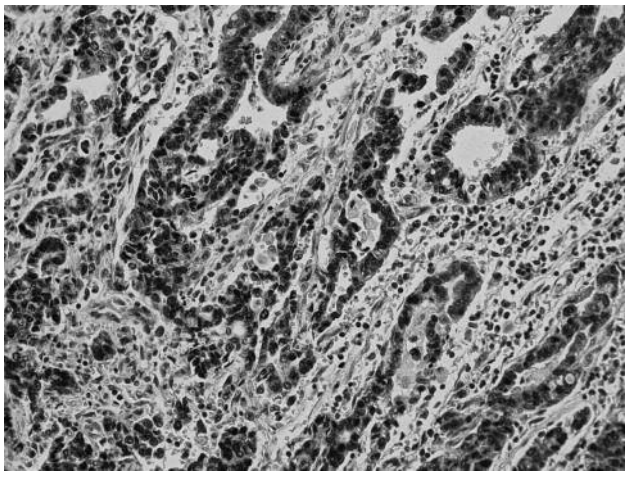

Figure 1. *Strong nuclear P15 staining in the majority of tumor cells (original magnification x400).*

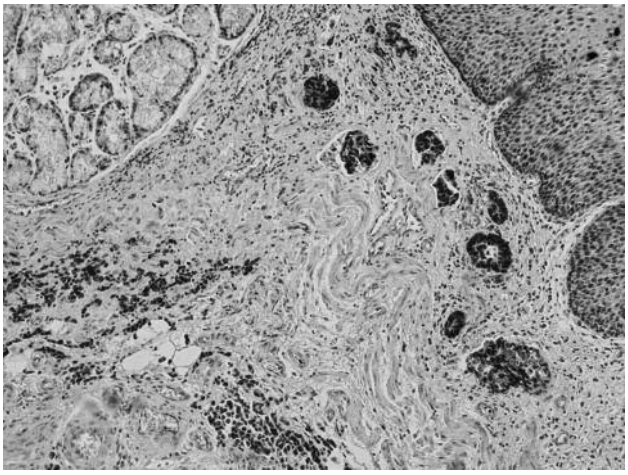

Figure 2. *Strong positive SMAD4 expression in the upper portions of a carcinoma invading the esophageal junction (original magnification x100).*

The median survival for all patients was 19 months (95% CI 16-22 months). The median survival was not found to be statistically related to the expression of P15 or SMAD4 ($p=0.194$ and $p=0.730$ respectively), nor to their co-expression ($p=0.689$) (data not shown). When the survival analysis was repeated stratifying by the degree of tumor differentiation (good or moderate *vs.* poor), the results for P15 did not change. However, in the case of well- or moderately differentiated tumors, survival was significantly better for patients with reduced SMAD4 expression *vs.* those with preserved SMAD4 expression ($p=0.042$) (Figure 3).

Table I. *P15 and SMAD4 expression in relation to clinicopathological data.*

	P15		*P*-value	SMAD4		*P*-value
	+	–		+ (Pre)	– (Red)	
Gender			**0.042**			0.597
Male	8 (20%)	33 (80%)		21 (49%)	20 (51%)	
Female	10 (45%)	12 (55%)		9 (41%)	13 (59%)	
Age (years)			0.999			0.613
36-70	10 (29%)	25 (71%)		18 (51%)	17 (49%)	
71-88	8 (29%)	20 (71%)		12 (43%)	16 (57%)	
Type			**0.047**			0.943
Intestinal	5 (15%)	28 (85%)		15 (46%)	18 (54%)	
Diffuse	7 (41%)	10 (59%)		9 (53%)	8 (47%)	
Mixed	6 (46%)	7 (54%)		6 (46%)	7 (54%)	
Differentiation			0.106			0.174
Good	1 (10%)	9 (90%)		2 (20%)	8 (80%)	
Moderate	2 (14%)	12 (86%)		7 (50%)	7 (50%)	
Poor	15 (39%)	24 (61%)		21 (54%)	18 (46%)	
Location			**0.041**			**0.012**
Proximal	8 (44%)	7 (16%)		12 (40%)	3 (9%)	
Distal	8 (44%)	24 (52%)		11 (37%)	21 (64%)	
Other	2 (11%)	14 (31%)		7 (23%)	9 (27%)	
Stage						
I or II	5 (36%)	9 (64%)	0.517	7 (50%)	7 (50%)	0.999
III or IV	13 (27%)	36 (73%)		23 (47%)	26 (53%)	

Pre: preserved, Red: reduced.

Discussion

Immunohistochemical expression of *p15* protein has never been evaluated in gastric cancer. The results of the present study lead us to certain speculations regarding the significance of *p15* protein expression in gastric carcinogenesis. The loss of *p15* protein expression was more frequently observed in the intestinal type of carcinoma, a finding that might imply an important role of p15 gene silencing in the development of this type of carcinoma. This hypothesis is in accordance with the observation already mentioned that the p15 gene is often hypermethylated in intestinal metaplasia, a precursor lesion of the intestinal type of gastric carcinoma (1, 14).

In our study, positive *p15* protein expression was not associated with better survival. This result might be due to the relatively small number of cases examined or to the advanced stage of the majority of the tumors, a possibility further supported by the suggestion that the loss of *p15* protein expression is an early event in gastric carcinogenesis (14). Moreover it could be hypothesized that *p15* protein expression does not actually play a significant role in the invasive or metastatic potential of gastric adenocarcinoma.

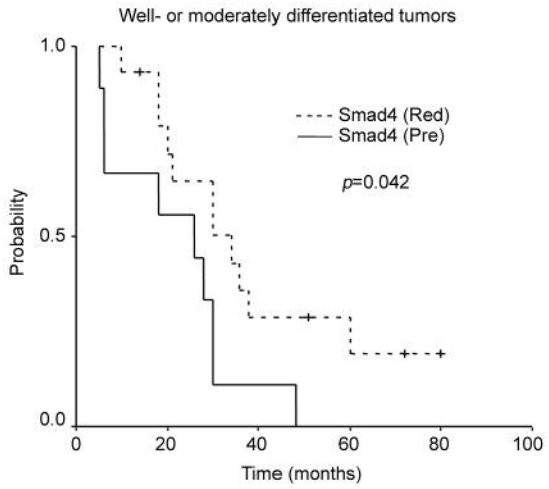

Figure 3. *Kaplan-Meier survival curves for patients with well- or moderately differentiated tumors. Patients with reduced SMAD4 expression (broken line) [median 34 months, 95% confidence interval (CI) 18-50 months] had a better survival rate than those with preserved SMAD4 expression (solid line) (median 26 months, 95% CI 3-49 months) (p=0.042).*

The immunohistochemical expression of *Smad4* protein has been studied recently in gastric carcinomas. The loss of *Smad4* protein expression has been shown to be significantly associated with advanced gastric cancer, depth of invasion (21-25) and poor prognosis (21, 23-25). In addition, by multivariate analysis, the reduction of *Smad4* expression was found to be an independent prognostic factor (21, 23).

In this study, no correlation between *Smad4* expression and survival was found when analysis included both low- and high-grade adenocarcinomas. However, when the survival of the patients was analyzed according to the degree of tumor differentiation, the patients with well- or moderately differentiated (low-grade) carcinomas had a significantly better outcome when their tumor showed reduced *Smad4* expression. These findings might be due to the different clone of SMAD4 antibody used compared to the other studies, or to the fact that most of our cases were stage III carcinomas (66.67%). Nevertheless, this finding might imply that *Smad4* plays different roles in the progression of gastric cancer, depending on the degree of tumor differentiation and aggressiveness. It is understood that TGF-β/Smad4 signaling has a biphasic nature in tumor development and that in a tumor microenvironment it can function in a growth-promoting fashion (26, 27). Based on this, we can speculate that in advanced gastric cancer, the TGF-β/Smad4 pathway that leads to the activation of *p15* and results in cell cycle arrest might be deregulated and, consequently, responsiveness to *Smad4* could be altered. Our results, indicating that reduced SMAD4 expression was correlated with better survival in low-grade tumors might imply that Smad4 expression gives an advantage to tumors regarding their invasive and metastatic potential. This could be achieved through induction of proteolytic activity, increased angiogenesis, or immunosuppression (28, 29). Further supporting this speculation is the report of Kim *et al.* (22) who stated that *Smad4* may be involved in a complex role in gastric carcinogenesis, behaving as a tumor suppressor in the early stages (gastric adenoma) and as a tumor promoter at later stages (adenocarcinoma).

It might also be possible that *Smad4* has only a limited role in gastric carcinogenesis. Previous studies have indicated that *Smad4* mutations are infrequent in gastric neoplasia (30). Moreover, in gastric carcinoma, no correlation has been proved between the loss of expression of *Smad4* protein and the LOH at 18q21 (31, 32). The latter observation highlights the possibility that there is another target of the LOH at this locus and that the gene involved might be *Smad7*, an inhibitory Smad that has recently been implicated in gastric carcinogenesis (24). Larger trials are necessary to test this hypothesis.

It has been reported that *p15* expression seems to be directly related to the presence of *Smad4* protein (19). In our series, a significant association was found between *p15* and *Smad4* protein expression, as 15 out of 18 (83%) P15-positive tumors also exhibited preserved SMAD4 expression ($p=0.001$). These immunohistochemical results are additional *in vivo* evidence supporting the direct relationship of these two proteins.

In conclusion, p15 gene silencing might be an early event in the development of adenocarcinomas of the intestinal type, while *Smad4* might act as a tumor promoter, in low-grade tumors. The observed association between *p15* and *Smad4* protein expression supports the inductive role of *Smad4* in *p15* transcription. Nevertheless, the results of our study imply that the Smad4/p15 pathway is probably deregulated in advanced gastric cancer. Restoration of its function could lead to inhibition of cellular proliferation and retardation of tumor growth.

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