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Loss of pS2 Protein Expression Is an Early Event of Intestinal-type Gastric Cancer

Ming-Shiang Wu,[1] Chia-Tung Shun,[2] Hsiu-Po Wang,[3] Wei-Jei Lee,[4] Teh-Hong Wang[1] and Jaw-Town Lin[1, 5]

Departments of [1]Internal Medicine, [2]Pathology, [3]Emergency Medicine and [4]Surgery, National Taiwan University Hospital, No. 7 Chun-Shan S. Rd., Taipei, Taiwan

To investigate the prevalence of pS2 expression in gastric cancer with respect to tumor histopathology, intestinal metaplasia and *Helicobacter pylori* (*H. pylori*) infection, pathologic specimens of 91 patients with gastric cancer were immunostained for pS2. Such immunoreactivity was correlated with the status of *H. pylori* infection, tumor staging, histology, subtyping, and associated intestinal metaplasia. Positive pS2 staining was seen throughout all non-neoplastic epithelia, and in all 9 patients with the complete type of intestinal metaplasia. In contrast, 21 of 45 incomplete type of intestinal metaplasia had negative pS2 staining ($P<0.001$), and 54 out of 91 tumors (59.3%) showed loss of pS2 expression in the cancer tissues proper. There was no correlation of pS2 expression with age, gender, depth of invasion, duodenal involvement, lymph node metastasis, venous invasion or *H. pylori* infection. Negative pS2 staining was significantly higher in the intestinal (74.5%) and Borrmann type I, II, III (64.2%) tumors than the diffuse (43.2%, $P<0.005$) and Borrmann type IV (20%, $P<0.05$) tumors. Our results indicate that loss of pS2 expression may occur as an early event in the malignant transformation process of intestinal-type tumors.

Key words: pS2 — Gastric cancer — Intestinal metaplasia — *Helicobacter pylori*

The gastrointestinal epithelium normally forms a barrier to intraluminal microorganisms and other noxious agents.[1)] In part, this defense is achieved by producing various peptides that may prevent injury and facilitate mucosal repair.[2)] Trefoil peptides, a new family of such peptides produced by goblet cells, may serve these functions.[3)] Among the three known trefoil peptides (pS2, PSP and ITF), pS2 has aroused a great deal of interest because of its potential role in tumorigenesis.[4)]

The pS2 gene, first discovered in a human breast carcinoma cell line, is also expressed in the normal stomach.[5, 6)] In view of the fact that increased expression of pS2 is noted in regions adjacent to alimentary tract ulceration, pS2 is thought to play a critical role in mucosal healing.[7, 8)] However, Lefebvre *et al*. have noted the absence of pS2 expression in mice with gastric mucosal dysplasia, adenomas, and intraepithelial carcinomas.[4)] This suggests that pS2 may also be involved in promoting epithelial cell proliferation in the process of tumorigenesis.

Several reports have since appeared showing pS2 expression in human gastric cancer (GC).[9–17)] The reported prevalence of pS2 immunoreactivity varied from 30 to 66 percent. Although pS2 expression is generally regarded as a marker of gastric-type differentiation, discrepant data exist concerning its expression, especially with respect to histologic subtypes, tumor stage and adjacent premalignant tissues. Furthermore, considering the important role of *Helicobacter pylori* (*H. pylori*) infection in epithelial injury and gastric carcinogenesis, it remains unclear whether *H. pylori* infection affects pS2 expression. To clarify these issues, we systematically studied the pS2 immunostaining in a series of GC tissues and correlated positive staining with tumor histopathology, intestinal metaplasia, and *H. pylori* infection, as well as other clinical and pathologic features.

MATERIALS AND METHODS

Patients and tissues A total of 91 patients, 52 males and 39 females with a mean age of 58.7 years (range: 31–81 years), with histologically confirmed GC were enrolled in this study. Their tumors were resected at the National Taiwan University Hospital between 1995 and 1996. No patient had received chemotherapy or radiation therapy before surgery. Preoperative serum was obtained immediately following endoscopy, and surgical specimens were handled according to the guidelines of the Japanese Research Society of GC.[18)] Tissues were fixed with neutral formalin, embedded in paraffin, and stained with hematoxylin and eosin. These tumors were classified into 47 intestinal type and 44 diffuse type on the basis of Lauren's criteria.[19)] The extent of tumor invasion was further subdivided into 10 early and 81 advanced.[18)] The mucosa adjacent to carcinoma, including intestinal metaplasia and the non-metaplastic epithelium, was also included for comparison. Intestinal metaplasia was categorized as complete or incomplete based on the presence or absence of mature absorptive cells, respectively.[20)] The status of *H.*

[5] To whom correspondence should be addressed.

pylori infection in each patient was determined by histological examinations on tissue sections of the non-tumorous specimen, as well as by the presence of a high titer of anti-*H. pylori* IgG in the preoperative serum.[21)]

Immunohistochemistry Immunostaining for pS2 protein was performed by using a standard avidin-biotin-peroxidase complex detection system. The monoclonal antibody used for this study was purchased from Signet Laboratory (Bedham, MA). In brief, 5 μm sections were dewaxed, microwaved, and rehydrated. Endogenous peroxidase activity and nonspecific bindings were blocked by incubation with 3% hydrogen peroxide (H_2O_2) and non-immune serum, respectively. The slides were then incubated sequentially at 4°C with the primary mouse monoclonal antibody overnight, a biotinylated goat anti-mouse secondary antibody for 30 min, peroxidase-conjugated streptavidin for 10 min and finally diaminobenzidine tetrachloride/H_2O_2 chromogen substrate for 10 min. Slides were then counterstained with Mayer's hematoxylin. Negative control sections were prepared by substituting the primary antibody with buffered saline, and positive control sections were obtained from breast carcinoma, known to express high levels of pS2. The percentage of positively stained cells was determined for each tumor section as well as its adjacent intestinal metaplasia and non-metaplastic epithelium. The immunostaining for pS2 was registered as negative only if less than 5% of the cells showed a positive staining.

Statistical analysis Comparison of categorical data such as the prevalence of pS2 expression between groups was performed by means of the two-tailed Fisher's exact test or χ^2 test. Numerical data expressed as mean±standard deviation were analyzed between groups by using the unpaired Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

In 54 (59.3%) of 91 resected tumors, intestinal metaplasia was observed in the epithelia adjacent to the tumor. Among those with intestinal metaplasia, 9 patients had the complete type and 45 patients had the incomplete type. A significantly higher frequency of intestinal metaplasia was found in the intestinal-type GC (40/47, 85.1%) than the diffuse-type GC (14/44, 31.8%, $P<0.001$). Positive pS2 staining was seen throughout the non-neoplastic epithelium in all specimens, mainly in the cytoplasm of antral mucopeptic glands and deep foveolar cells of the gastric pits (Fig. 1). Loss of pS2 expression was not found in any of the 9 patients with the complete-type intestinal metaplasia, while 21 (46.7%) of 45 patients with the incomplete-type intestinal metaplasia showed negative

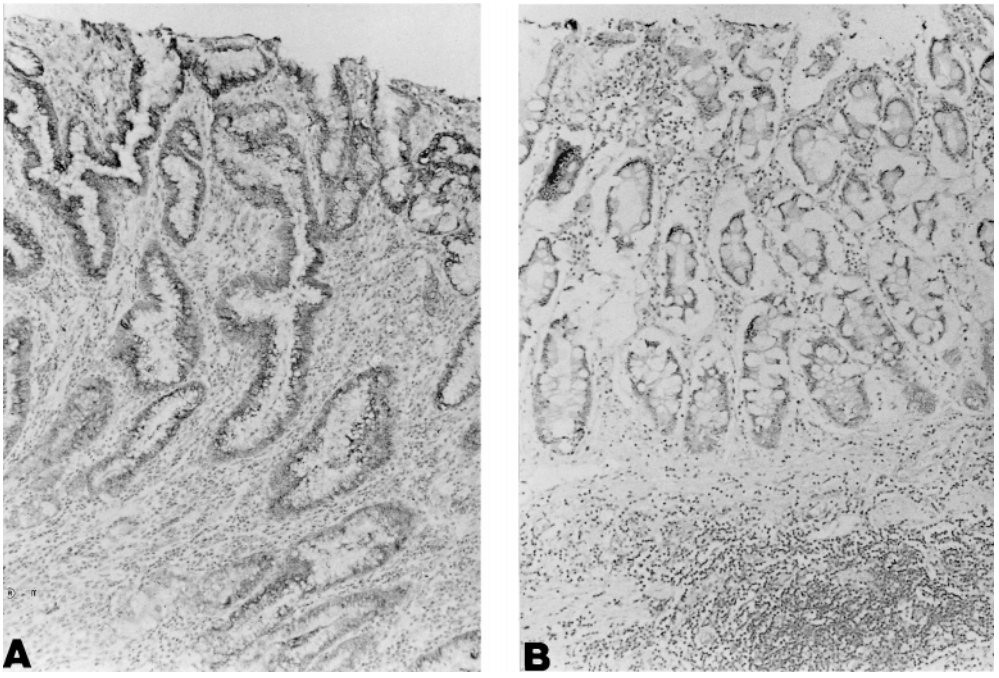

Fig. 1. A, Positive pS2 immunoreactivity (brownish color) is seen throughout the normal gastric mucosa, mainly in the cytoplasm of superficial and foveolar epithelium (100×); B, A case of incomplete intestinal metaplasia with negative pS2 staining (100×).

Table I. The Prevalence of pS2 Immunoreactivity in 91 Patients with Gastric Cancer in Relation to Different Clinicopathologic Features

Variable	pS2 expression	
	negative (*n*=54)	positive (*n*=37)
Mean age (years)	59.5±13.1	57.6±12.3
Gender		
Male	33	19
Female	21	18
Histologic type		
Diffuse[a)]	19	25
Intestinal	35	12
Borrmann type		
I	4	1
II	10	8
III	38	20
IV[b)]	2	8
Tumor location		
Cardia	5	2
Noncardia	49	35
Depth of invasion		
Early	6	4
Advanced	48	33
Duodenal involvement		
Positive	11	6
Negative	43	31
Lymph node metastasis		
Positive	34	20
Negative	20	17
Venous invasion		
Positive	18	10
Negative	36	27
Helicobacter pylori infection		
Histology		
Positive	38	22
Negative	16	15
Serology		
Positive	40	23
Negative	14	14

a) $P<0.005$. *b*) $P<0.05$.

expression ($P<0.01$). In carcinomatous tissues, pS2 staining was negative in 54 (59.3%) of 91 tumors examined. The data on pS2 immunoreactivity in relation to different clinicopathologic features are summarized in Table I. No statistically significant correlation of pS2 expression with age, gender, depth of invasion, duodenal involvement, lymph node metastasis, venous invasion, or *H. pylori* infection was noted. Negative pS2 staining was significantly higher in the intestinal (35/47, 74.5%) and Borrmann type I, II, III (52/81, 64.2%) tumors than in the diffuse (19/44, 43.2%, $P<0.005$) and the Borrmann type IV (2/10, 20%, $P<0.05$) tumors.

DISCUSSION

Gastric carcinogenesis is a multistep process associated with multiple genetic alterations.[22)] Various genetic abnormalities, including activation of oncogenes, inactivation of tumor suppressor genes and dysfunction of mismatch repair genes, have been reported in GC.[23)] Several lines of evidence have recently suggested that pS2 may be involved in gastric tumorigenesis.[4, 9–17)] Low or undetectable expression of pS2 mRNA is documented in GC cell lines and tumor samples.[9, 17)] Furthermore, the absence of pS2 in mice leads to the formation of gastric adenoma and adenocarcinoma.[6)] These studies clearly indicated that loss of pS2 expression may be involved in altered growth control of tumor cells.[6, 9, 17)] However, it remains unclear whether loss of pS2 expression can be correlated with different clinicopathologic status of GC.

Using immunohistochemistry, we observed positive pS2 immunoreactivity in foveolar and mucus neck cells of the normal gastric mucosa, a finding consistent with other reports.[9–17)] In contrast, positive pS2 immunostaining was found in only 40.7% of GC tissues, which falls in the reported range of frequency in GC, i.e., 30% to 66%, without taking into consideration different histologic subtyptes.[12, 15)] Such variation in the frequency of pS2 immunoreactivity may be due to different geographic populations or different antibodies and criteria adopted in previous studies.[9–17)]

While only one report found no relationship between pS2 expression and histologic subtypes of GC,[12)] all other recent data suggest that there is a close association between intestinal-type GC and a higher percentage of negative pS2 expression.[11, 14)] In knockout mice, loss of pS2 results in development of adenomas and well-differentiated GC.[4)] Our result also showed a significantly higher rate of loss of pS2 immunostaining in the intestinal-type and Borrmann type I, II, and III cancers. We surmise that loss of pS2 expression favors the development of more differentiated structures, i.e., the intestinal-type malignancies. Because ultrastructural studies revealed that the majority of diffuse carcinoma cells are gastric-type cells (foveolar and/or mucopeptic cells), it is assumed that pS2 expression has a major impact on the proliferation and differentiation of gastric epithelial cells.[3, 24, 25)] This notion is supported by a strong correlation between expression of pS2 and markers of gastric differentiation, such as pepsinogen II and 2B5.[12)] However, the exact function of pS2 remains ill-defined and the molecular basis of pS2 function in promoting tumorigenesis deserves further study.

One approach would be to examine whether pS2 alteration occurs during the progression of gastric carcinogenesis. Some reports have shown that pS2 expression is correlated with extent of tumor growth and lymph node

metastasis, implying a role of pS2 in the late stage of tumor progression.[12, 14] Nevertheless, other reports have presented contradictory findings. Lack of correlation of pS2 expression with most features of tumor aggressiveness, such as depth of invasion, tumor stage, lymph node metastasis, and venous invasion, has been documented by Machado *et al*.[14] In our series, no correlation could be found between the pS2 expression and age, gender, duodenal involvement, lymph node metastasis, or venous invasion of GC. Similar frequencies of pS2 immunoreactivity were also observed in early and advanced GC. In addition to dietary factors, *H. pylori* infection has recently been recognized as the most important environmental factor for GC.[26] It is speculated that *H. pylori*-associated inflammation may enhance cellular proliferation and favor genetic alterations, albeit no alteration has yet been identified.[27] In this study, a higher frequency of pS2 loss in GC with positive *H. pylori* infection was noted, but this was without statistical significance. Further studies are needed to clarify the role of *H. pylori* infection in pS2 alteration in gastric tumorigenesis.

Previous studies of pS2 expression in gastrointestinal tract have detected pS2 in normal epithelium of stomach, jejunum, ileum, and epithelium adjacent to areas of mucosal damage or colonic tumors.[10, 28] However, no pS2 expression was noted in normal epithelium of duodenum or colon. The factors controlling pS2 expression and distribution in the gastrointestinal tract are unclear.[28] Intriguingly, pS2 alteration might be involved in premalignant change in gastric carcinogenesis. This was supported by investigations on pS2 expression in intestinal metaplasia, a premalignant lesion of GC.[20, 29] Recent studies have indicated that incomplete or colonic-type intestinal metaplasia, characterized by incomplete cell differentiation, has a more selective association with GC.[29] In 91 resected specimens, we found that 85.1% of intestinal GC and 31.8% of diffuse GC were associated with intestinal metaplasia. In 54 patients with intestinal metaplasia, loss of pS2 expression was more frequently encountered in the incomplete type (46.7%) than in the complete type (0%). According to Correa's model of gastric carcinogenesis,[22] such an observation might have the following three implications. First, different frequencies of intestinal metaplasia in these two subtypes of GC support the existence of at least two distinct pathways of malignant transformation of gastric mucosa. Secondly, the incomplete type of intestinal metaplasia is a more advanced lesion than the complete type because loss of pS2 expression is more frequently noted in the former. Thirdly, loss of pS2 expression may be used as a potential marker for a premalignant lesion of GC.

In conclusion, our results suggest that pS2 expression is correlated with different histologic subtypes of GC and its loss may occur as an early event, especially in the malignant transformation of intestinal-type GC.

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