Asporin Expression on Stromal Cells and/or Cancer Cells Might Be A Useful Prognostic Marker in Patients with Diffuse-Type Gastric Cancer

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Keywords

Asporin · Stromal cells · Gastric cancer

Abstract

Background: Asporin (ASP), a member of the proteoglycan family, has been shown to have a close correlation with cancer progression. It is not known whether ASP is an oncogenic driver or a tumor suppressor in human gastric cancer. We sought herein to determine the relationship between ASP expression and clinicopathological features of gastric cancer. **Patients and Methods:** A total of 296 gastric cancer patients (diffuse type, $n = 144$; intestinal type, $n = 152$) were enrolled. The ASP expression level in each case was analyzed by immunohistochemistry. **Results:** ASP was mainly found on stromal cells, especially on fibroblasts in tumor stroma, i.e., cancer-associated fibroblasts. The ASP expression on either cancer cells or stromal cells was significantly

high in macroscopic scirrhous-type tumors ($p < 0.001$) and histologically abundant stroma-type tumors ($p < 0.001$). Interestingly, a Kaplan-Meier survival curve of the 144 cases of diffuse-type gastric cancer revealed a significantly poorer prognosis in patients with ASP-positive expression ($p = 0.043$; log rank) compared to those with ASP-negative expression, but the prognoses were not significantly different in these subgroups of the 152 cases of intestinal-type gastric cancer. A multivariate analysis with respect to overall survival showed that ASP expression on stromal cells and/or cancer cells was significantly correlated with overall survival in patients with diffuse-type gastric cancer ($p = 0.041$). **Conclusion:** In gastric cancer, ASP was expressed mainly on stromal cells and partially on cancer cells. ASP expression on stromal cells and/or cancer cells might be a useful prognostic marker in patients with diffuse-type gastric cancer.

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Introduction

Asporin (ASPIN) has been recognized as a member of the family of class I small leucine-rich repeat proteoglycans and a major noncollagen component of the extracellular matrix [1–3]. It was recently reported that there is a close correlation between ASPIN and cancer progression [4]. ASPIN might play a negative role or a positive role in the pathogenesis of different cancers, because ASPIN expression has been reported to be positively or negatively correlated with cancer proliferation and migration by regulating different signaling pathways in each cancer type [4]. For example, ASPIN acts as an oncogene in some types of cancer, including gastric cancer [5, 6], pancreatic cancer [7, 8], colorectal cancer [9], and prostate cancer [10], whereas ASPIN acts as a tumor suppressor gene in triple-negative breast cancer [11–13].

It was also reported that ASPIN is produced in cancer cells or stromal cells, and most of the relevant studies showed that ASPIN is produced from stromal cells in the tumor microenvironment [4]. We demonstrated that ASPIN secreted from cancer-associated fibroblasts (CAF) activates the invasion activity of scirrhous gastric cancer cells, one of the specific types of diffuse-type gastric cancer [5], via paracrine effects. However, it is not known whether ASPIN is an oncogenic driver or a tumor suppressor in human gastric cancer (including diffuse-type cancer and intestinal-type cancer). We conducted the present study to determine the relationship between ASPIN expression and clinicopathological features of gastric cancer, including diffuse-type cancer and intestinal-type cancer.

Materials and Methods

Patients

We examined surgical samples from patients at the Osaka City University Hospital, Osaka, Japan. A total of 296 patients who had undergone resection of primary gastric tumors and were confirmed histologically to have gastric cancer were enrolled in this study. None of the patients had undergone preoperative radiation or chemotherapy. The median follow-up time for all 296 patients was 48.2 months (range 2–90). The median follow-up time for the patients who died of the disease was 19.9 months, compared to 60.5 months for surviving patients. The follow-up schedule after gastrectomy was managed according to the Japanese guideline [14], as follows: computed tomography (CT) was performed at intervals of 6–12 months, measurement of tumor markers of CEA and CA19-9 was performed at intervals of 3–6 months, and endoscopy was performed at intervals of 2 years.

Pathological diagnoses and classifications were made according to the Japanese classification of gastric carcinoma (14th edi-

tion) [15]. Depth of tumor invasion, histological type, lymph node metastasis, venous invasion, and lymphatic invasion were based on microscopic examination of materials obtained by surgical resection. Histological subtypes were classified as intestinal and a diffuse; the intestinal type includes papillary adenocarcinoma, well-differentiated tubular adenocarcinoma, and moderately-differentiated adenocarcinoma, and the diffuse type includes poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous carcinoma.

Immunohistochemical Techniques

Paraffine-embedded sections were deparaffinized in xylene and dehydrated through graded ethanol. The sections were heated for 4 min at 125 °C by autoclave in Target Retrieval Solution (DAKO, Carpinteria, CA, USA). Then the sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were then incubated with anti-ASPIN antibody (HPA008435, 1: 750; Sigma Life science, St. Louis, MO, USA) for 30 min at room temperature. The sections were incubated with biotinylated goat anti-rabbit immunoglobulin G for 30 min, followed by 3 washes with PBS. The slides were treated with streptavidin-peroxidase reagent and incubated in PBS diaminobenzidine and 1% hydrogen peroxide (volume/volume), followed by counterstaining with Mayer hematoxylin. The Anti-ASPIN antibody (HPA008435) used in this study was validated in 2 independent studies for immunohistochemistry [5, 16].

Immunohistochemical Determination

ASPIN expression was evaluated on cancer cells and stromal cells individually based on the intensity of staining and the extent of staining at the invading tumor front. An intensity value of 0 denoted undetectable staining, whereas values of 1 and 2 denoted a low and high immunoreactivity, respectively. Regarding the extent of staining, values of 1 or 2 denoted samples in which < 50% or 50% ≥ of the tissue showed immunoreactivity correspondingly. The results obtained using these 2 scales were then added together, yielding a single value from 0 to 4, i.e., the score. In both cancer cells and stromal cells, the cut-off point was determined to be 2; ASPIN expression was considered positive when scores were ≥3 and negative when scores were ≤2. The evaluation was made by 2 independent observers without knowledge of the clinical data and histologic diagnoses. When a discrepant evaluation between the 2 independent observers was found, the evaluation was rechecked and discussed and the final score was obtained by consensus.

Statistical Analysis

The statistical significance of the associations between ASPIN expression and various clinicopathological variables was evaluated using a χ^2 test or the Fisher exact test. The survival durations were calculated using the Kaplan-Meier method and analyzed by the log-rank test to compare the cumulative survival durations in the patient groups. In addition, the Cox proportional hazards model was used to compute univariate and multivariate HR for the study parameters. In all of the tests, $p < 0.05$ was considered statistically significant. SPSS software (SPSS Japan, version 22; Tokyo, Japan) was used for the analysis.

Fig. 1. ASPN expression in diffuse-type cancer and intestinal-type cancer. ASPN was expressed at the cytosol of cells in the gastric tumor environment. In both diffuse-type cancer (top) and intestinal-type cancer (bottom), ASPN was expressed on cancer cells and/or stromal cells, especially on fibroblasts.

Fig. 2. Survival of patients with gastric cancer. a Kaplan-Meier survival curve for all 296 patients; no correlation was observed between the ASPN-positive and ASPN-negative groups ($p = 0.819$). b Among the 144 patients with diffuse-type cancer, the prognosis of patients with ASPN-positive expression was significantly poorer than that of patients with ASPN-negative expression ($p = 0.043$). c Among the 152 cases of intestinal-type gastric cancer, ASPN expression was not correlated with the prognosis ($p = 0.104$).

Results

The Relationship between Clinicopathologic Features and ASPN Expression

ASPN was expressed on either cancer cells or stromal cells in the gastric tumor environment (Fig. 1). ASPN was

present mainly on stromal cells, especially on fibroblasts in tumor stroma, i.e., CAF. Some gastric tumors expressed ASPN on both cancer cells and stromal cells. Among the 296 cases of gastric cancer, 17 cases (5.8%) showed ASPN-positive expression on cancer cells and 72 cases (24.3%) showed ASPN-positive expression on stro-

Table 1. Correlation between ASPN expression on cancer cells and/or stromal cells and clinicopathologic features in 296 patients with gastric carcinoma

Clinicopathologic features	ASPN expression					
	cancer cells		stromal cells		either cancer cells or stromal cells	
	positive (n = 17)	negative (n = 277)	p value	positive (n = 72)	negative (n = 224)	p value
Age (years)						
<65	11 (6.9)	149 (93.1)		40 (24.8)	121 (75.2)	
≥65	6 (4.5)	128 (95.5)	0.380	32 (23.7)	103 (76.3)	0.820
Gender						
Female	6 (6.7)	83 (93.3)		26 (28.9)	64 (71.1)	
Male	11 (5.4)	194 (94.6)	0.642	46 (22.3)	160 (77.7)	0.226
Macroscopic type						
Scirrhus	1 (3.0)	32 (97.0)		18 (54.5)	15 (45.5)	
Other	16 (6.1)	245 (93.9)	0.409	54 (20.5)	209 (79.5)	<0.001
Depth of invasion						
T1, 2	6 (4.7)	123 (95.3)		37 (28.5)	93 (71.5)	
T3, 4	11 (6.7)	154 (93.3)	0.463	35 (21.1)	131 (78.9)	0.142
Lymph node metastasis						
Negative	11 (6.8)	151 (93.2)		37 (22.7)	126 (77.3)	
Positive	6 (4.7)	123 (95.3)	0.440	33 (25.4)	97 (74.6)	0.592
Stages						
I and II	13 (6.8)	178 (93.2)		43 (22.4)	149 (77.6)	
III and IV	4 (3.9)	98 (96.1)	0.314	29 (28.2)	74 (71.8)	0.272
Histological type						
Differentiated	13 (8.6)	138 (91.4)		37 (24.3)	115 (75.7)	
Undifferentiated	4 (2.8)	139 (97.2)	0.044	35 (24.3)	109 (75.7)	0.994
Cancer stromal volume						
Med/Int	9 (5.1)	165 (94.9)		26 (14.9)	149 (85.1)	
Sci	4 (5.3)	72 (94.7)	0.968	33 (42.9)	44 (57.1)	<0.001
Lymphatic invasion						
Negative	11 (8.3)	121 (91.7)		35 (26.5)	97 (73.5)	
Positive	6 (3.7)	155 (96.3)	0.093	37 (22.7)	126 (77.3)	0.448
Venous invasion						
Negative	12 (4.8)	236 (95.2)		62 (24.9)	187 (75.1)	
Positive	5 (10.9)	41 (89.1)	0.108	10 (21.3)	37 (78.7)	0.595
Hepatic metastasis						
Negative	17 (5.9)	271 (94.1)		72 (24.8)	218 (75.2)	
Positive	0 (0)	6 (100)	0.540	0 (0)	6 (100)	0.161
Peritoneal metastasis						
Negative	17 (6.1)	260 (93.9)		66 (23.7)	213 (76.3)	
Positive	0 (0)	17 (100)	0.293	6 (35.3)	11 (64.7)	0.278
Peritoneal cytology						
Negative	13 (5.7)	215 (94.3)		48 (21.0)	181 (79.0)	
Positive	1 (2.7)	36 (97.3)	0.700	14 (37.8)	23 (62.2)	0.024

Values are presented as numbers (%). Med, medullary type; scanty stroma; Sci, scirrhus type (sci); abundant stroma; Int, intermediate type; the quantity of stroma is intermediate between the 2 above types.

Table 2. Univariate analysis and multivariate analysis with respect to overall survival in 296 patients with gastric carcinoma

Parameter	Univariate analysis			Multivariate analysis		
	risk ratio	95% CI	<i>p</i> value	risk ratio	95% CI	<i>p</i> value
ASPN expression on cancer cells and/or stromal cells						
Negative vs. positive	1.056	0.665–1.676	0.820			
ASPN expression on stromal cells						
Negative vs. positive	1.048	0.647–1.697	0.849			
ASPN expression on cancer cells						
Negative vs. positive	1.098	0.479–2.515	0.825			
Age (years)						
<65 vs. ≥65	1.713	1.114–2.635	0.014	1.241	0.776–1.984	0.367
Gender						
Female vs. male	1.096	0.696–1.728	0.692			
Histological type						
Intestinal diffuse	1.832	1.184–2.834	0.007	1.429	0.749–2.726	0.279
Macroscopic type						
Scirrhou vs. other	9.091	5.663–14.594	<0.001	6.910	3.187–14.981	<0.001
Invasion depth (T stage)						
I/II vs. III/IV	5.323	3.312–8.555	<0.001	1.762	0.941–3.298	0.077
Lymph node metastasis						
Negative vs. positive	6.884	4.096–11.568	<0.001	5.264	2.093–13.242	<0.001
Hepatic metastasis						
Negative vs. positive	8.131	3.518–18.793	<0.001	3.361	0.988–11.437	0.052
Peritoneal dissemination						
Negative vs. positive	7.943	4.508–13.993	<0.001	3.138	1.469–6.706	0.003

mal cells. Eighty cases (27%) showed ASPN-positive expression on either cancer cells or stromal cells, and 9 cases (3%) showed the expression on both cancer cells and stromal cells.

The relationship between ASPN expression on cancer cells and/or stromal cells and clinicopathological features is described in Table 1. ASPN expression on stromal cells was significantly high in macroscopic scirrhou-type tumors ($p < 0.001$), histologically abundant stroma-type tumors ($p < 0.001$), and peritoneal cytology ($p = 0.024$). ASPN expression on cancer or stromal cells was also significantly high in macroscopic scirrhou-type tumors ($p < 0.001$) and histologically abundant stroma-type tumors ($p < 0.001$). In contrast, there was no significant correlation between ASPN expression on cancer cells and any of the clinicopathological features.

Prognosis

Figure 2a shows the Kaplan-Meier survival curve for all 296 patients; no significant difference in prognosis was observed between the ASPN-positive and ASPN-negative groups ($p = 0.819$). Gastric cancer is histologically classified into 2 types by the Lauren classification as follows: diffuse type and intestinal type. Interestingly, in the 144

cases of diffuse-type gastric cancer, significantly poorer prognoses were observed in patients with ASPN-positive expression compared to those with ASPN-negative expression ($p = 0.043$; Fig. 2b), whereas in the 152 cases of intestinal-type gastric cancer the prognoses of patients with ASPN-positive expression were significantly better than those of patients with ASPN-negative expression ($p = 0.104$; Fig. 2c).

The results of the univariate analysis with respect to overall survival for all 296 patients indicated that ASPN expression on cancer cells or stromal cells was not significantly associated with a poorer prognosis, whereas age ≥ 65 years ($p = 0.014$), histologic diffuse type ($p = 0.007$), macroscopic scirrhou type ($p < 0.001$), invasion depth ($p < 0.001$), lymph node metastasis ($p < 0.001$), hepatic metastasis ($p < 0.001$), and peritoneal dissemination ($p < 0.001$) were significantly associated with a poor prognosis. In the univariate analysis, macroscopic scirrhou type ($p < 0.001$), lymph node metastasis ($p < 0.001$), and peritoneal dissemination ($p = 0.003$) were significantly associated with a poor prognosis (Table 2). Table 3 provides the results of the univariate analysis and multivariate analysis with respect to the overall survival of the 144 diffuse-type gastric cancer patients. In the univariate analy-

Table 3. Univariate analysis and multivariate analysis respect to overall survival in 144 diffuse-type gastric cancer patients

Parameter	Univariate analysis			Multivariate analysis		
	risk ratio	95% CI	<i>p</i> value	risk ratio	95% CI	<i>p</i> value
ASP expression on cancer cells and/or stromal cells						
Negative vs. positive	2.126	1.041–4.340	0.038	1.686	1.020–2.786	0.041
ASP expression on stromal cells						
Negative vs. positive	1.811	1.011–3.246	0.055			
ASP expression on cancer cells						
Negative vs. positive	1.423	0.346–5.853	0.625			
Age (years)						
<65 vs. ≥65	1.588	0.915–2.755	0.100			
Gender						
Female vs. male	1.969	1.080–3.589	0.027	0.991	0.617–1.592	0.970
Macroscopic type						
Scirrhou vs. other	9.313	5.170–16.779	<0.001	4.609	2.602–8.164	<0.001
Invasion depth (T stage)						
I/II vs. III/IV	7.862	3.689–16.753	<0.001	1.762	0.941–3.298	0.077
Lymph node metastasis						
Negative vs. positive	11.128	4.733–26.166	<0.001	2.829	1.487–5.383	0.002
Hepatic metastasis						
Negative vs. positive	5.632	1.749–18.140	<0.001	3.225	1.328–7.832	0.010
Peritoneal dissemination						
Negative vs. positive	8.381	4.149–14.747	<0.001	3.164	1.328–7.832	<0.001

sis, ASPN expression on cancer cells and/or stromal cells ($p = 0.038$), male gender ($p = 0.027$), macroscopic scirrhou type ($p < 0.001$), invasion depth ($p < 0.001$), lymph node metastasis ($p < 0.001$), hepatic metastasis ($p < 0.001$), and peritoneal dissemination ($p < 0.001$) were significantly associated with a poor prognosis. The results of the multivariate analysis showed that ASPN expression ($p = 0.041$), macroscopic scirrhou type ($p < 0.001$), lymph node metastasis ($p = 0.002$), hepatic metastasis ($p = 0.010$), and peritoneal dissemination ($p < 0.001$) were significantly correlated with overall survival in patients with diffuse-type gastric cancer.

Discussion

In the present study, ASPN was expressed on cancer cells and/or stromal cells in gastric tumors. It was unclear which types of cells expressed ASPN in tumor microenvironments. ASPN has been reported to be expressed mainly on stromal cells, especially CAF, in gastric cancer [5, 6, 17], pancreatic cancer [7, 8], colorectal cancer [9], lung cancer [18], and prostate cancer [10, 16]. ASPN expression has also been detected on cancer cells of triple-negative breast cancer [11–13] and colorectal cancer [9]. Our present analyses demonstrated that ASPN was ex-

pressed on cancer cells and/or stromal cells in 80 (27%) of the 296 gastric cancers. Among these 80 cases, 72 cases and 17 cases were ASPN-positive on stromal cells and cancer cells, respectively, and 9 cases were ASPN-positive on both cells. These ASPN expression cases were significantly frequent in macroscopic scirrhou-type tumors, in abundant stroma tumors, and in peritoneal dissemination.

A characteristic of scirrhou-type gastric cancer is rapid proliferation with abundant stromal cells [19] and a high incidence of peritoneal dissemination [20, 21]. Because normal gastrointestinal tissues do not express ASPN [8], ASPN might be produced mainly by cancer-specific stromal cells such as activated fibroblasts, i.e., CAF. Those findings indicated that ASPN from CAF might be associated with the phenotype of scirrhou gastric cancer, one of the specific types of diffuse-type gastric cancer.

Gastric cancer is histologically classified into a diffuse type or an intestinal type by the Lauren classification [22]. In diffuse gastric cancer, cancer cells grow by poor adhesion, and a few cells infiltrate into the stroma, with the result that cancer cells are surrounded by stromal cells. Although our present analyses showed that prognoses were not significantly different between the ASPN-positive and ASPN-negative groups among the 296 patients,

a significant difference in prognosis was revealed between these 2 groups among the 144 patients with diffuse-type gastric cancer. On the other hand, among those 144 cases of diffuse-type gastric cancer, a significantly poorer prognosis was observed in patients with ASPN-positive expression compared to those with ASPN-negative expression, but the prognoses of patients with ASPN-positive tumors were not significantly between these 2 groups among the 152 patients with intestinal-type gastric cancer.

It has been reported that ASPN promotes cancer cell proliferation and invasion by binding with CD44 or transforming growth factor (TGF)- β /Smad [4, 9], which are frequently expressed in diffuse-type gastric cancer. Our prior investigation indicated that the gastric cancer cell line 44As3 promotes ASPN expression in CAF via a mechanism that functions through a CD44/Rac1-mediated axis [5]. ASPN has been reported to act as a negative regulator of TGF β /Smad signaling, which shows suppressive effects against the proliferation of gastric cancer cells [23–27]. These findings suggest that ASPN might stimulate the progression of diffuse-type gastric cancer cells with CD44 or TGF β 1/Smad expression. ASPN might be a valuable therapeutic target in patients with diffuse-type gastric cancer.

Our multivariate analysis in patients with diffuse-type gastric cancer revealed that ASPN expression was an independent prognostic factor. ASPN from CAF may play a critical role in the progression of diffuse-type gastric cancer with CD44 or TGF β 1/Smad expression, resulting in a poor prognosis for patients with ASPN-positive expression.

We also identified 17 gastric cancer cases that had ASPN-positive expression on cancer cells. There was no significant correlation between ASPN expression on cancer cells and any of the clinicopathological features. ASPN was expressed on gastric cancer cells in relatively few cases, and it thus might not play an important role in the phenotype of gastric cancer.

One of limitations of this study is the histologic heterogeneity of gastric carcinoma. Gastric carcinoma frequently shows the histologic heterogeneity with coexistence of intestinal-type and diffuse-type adenocarcinomas, and the histologic type is determined based on the dominant element of histologic patterns as well as this study. The significance of ASPN in gastric cancer with histologic heterogeneity must be studied in the future. The other limitation of this study is the effect of postoperative chemotherapy. This study included GC patients in an advanced stage who underwent adjuvant or postop-

erative chemotherapy. Since an analysis of the effect of chemotherapy would be important, we would like to examine the significance of postoperative chemotherapy in a future study.

In conclusion, ASPN was expressed mainly on stromal cells and partially on cancer cells in gastric cancer. ASPN expression on stromal cells and/or cancer cells might be a useful prognostic marker in patients with diffuse-type gastric cancer.

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Statement of Ethics

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki. This study was approved by the Osaka City University Ethics Committee (reference No. 924). Informed consent was obtained from all of the patients prior to entry.

Conflict of Interest Statement

There are no financial or other interests with regard to this paper that might be construed as a conflict of interests.

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Author Contributions

M.Y. and T.H. designed and performed the experiments, cowrote this paper, and contributed equally. T.F., Y.Y., and N.K. were responsible for sample preparation. K.S., M.Y., A.S., S.K., S.N. and T.T. were responsible for data accumulation. G.T., N.K., and M.O. did the material sampling. M.O. reviewed this paper. All of the authors read and approved the final version of this work.

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