

# Soluble podoplanin as a biomarker in diffuse-type gastric cancer

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**Abstract.** Diffuse-type gastric cancer, also known as scirrhous gastric cancer, is characterized by a larger number of stromal cells, referred to as cancer-associated fibroblasts (CAFs), than the number of cancer cells in the tissue. The present study focused on CAFs in gastric cancer and examined their potential as a blood biomarker. A total of 46 and 84 patients with gastric cancer were respectively included in a development and an independent validation cohort to assess the clinicopathological characteristics of plasma podoplanin (PDPN) levels. The prognostic impact of plasma PDPN was also investigated in the validation cohort. The cut-off value of the plasma-PDPN concentration was set to the median plasma PDPN concentration in the development cohort that was then divided into the high-PDPN and low-PDPN groups. The high-PDPN group tended to have more diffuse-type disease ( $P=0.079$ ), which was further confirmed through logistic regression analysis ( $P=0.008$ ). Kaplan-Meier survival estimates indicated that the recurrence-free survival rate was significantly lower in the high-PDPN group ( $P=0.029$ ). In conclusion, plasma soluble PDPN was demonstrated to be a marker for diffuse gastric cancer and may reflect the prognosis of this disease.

## Introduction

Gastric cancer is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related death (1). Various biomarkers targeting gastric cancer cells have been studied; however, there are no standardized and established biomarkers, particularly for scirrhous gastric cancer (2-4). A large amount of stroma containing fibroblasts is present around scattered

gastric cancer cells; this feature is remarkable in scirrhous gastric cancer, which, among the subtypes of gastric cancer, has a poor prognosis (5,6). Thus, an analysis that focuses solely on the expression in cancer cells themselves may not be sufficient to identify biomarkers that reflect the condition of patients with scirrhous gastric cancer.

The stromal cells around a cancer lesion are called cancer-associated fibroblasts (CAFs) and it has been reported that CAFs are involved in cancer progression and the metastatic potential of gastric cancer (7,8). There are several CAF markers in gastric cancer, including  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), fibroblast activation protein (FAP) and podoplanin (PDPN) (9,10). Previous research by our group focused on PDPN, which is expressed in lymphatic endothelium, as a CAF marker in gastric cancer and the results indicated that high PDPN expression in CAFs is a poor prognostic factor in patients with gastric cancer (11). Furthermore, it was observed that PDPN is expressed in CAFs but rarely in gastric cancer cells (11). In the present study, it was hypothesized that it may be useful to focus on molecules present in gastric cancer stromal tissue rather than gastric cancer cells as biomarkers to indicate the pathology of scirrhous gastric cancer.

Using liquid biopsy instead of tissue biopsy may enable a comprehensive evaluation of patient pathology. The present study aimed to use liquid biopsy to search for biomarkers targeting CAFs. In addition, the clinical significance of soluble PDPN was investigated in patients with gastric cancer by assessing the relationship between plasma PDPN levels and patients' clinicopathological factors and prognosis. Furthermore, the biological significance of soluble PDPN was examined by assessing the association between tissue and plasma PDPN expression and by evaluating the function of soluble PDPN *in vitro*.

## Materials and methods

**Patients and samples.** The present study was performed as a joint research project of the Faculty of Medicine, University of Yamanashi (Yamanashi, Japan) and the Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine (Kyoto, Japan). A total of 46 and 84 patients with gastric cancer were respectively included in a development

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cohort (Table I) and an independent validation cohort (Table SI) to assess the clinicopathological characteristics of plasma PDPN levels. Furthermore, the analysis was performed using the data of all 130 patients from both cohorts (Table II). The development cohort included patients who underwent radical resection at Yamanashi University Hospital (Yamanashi, Japan) between December 2017 and May 2020. The independent validation cohort included patients with stage I-III gastric cancer who underwent radical resection at Kyoto Prefectural University of Medicine Hospital (Kyoto, Japan) between June 2010 and December 2015. The prognostic impact of plasma PDPN expression was also investigated by determining the 5-year overall survival (OS) rate and 5-year the recurrence-free survival (RFS) rate in the validation cohort with stage I-III gastric cancer. Patient demographic data and details of tumor recurrence and subsequent management were recorded. The pathological classification of tumors was determined according to the Union for International Cancer Control classification (12).

*Preparation of plasma samples.* From each patient, a 5-ml blood sample was collected in an EDTA tube prior to surgery. Plasma was immediately separated from the cellular fraction by centrifugation as described elsewhere (13) and then stored at -80°C for further processing.

*Plasma PDPN analysis using ELISA.* Quantification of PDPN expression in the plasma was performed using the Human Podoplanin ELISA kit (cat. no. ELH-PDPN-1; RayBiotech, Inc.) according to the manufacturer's protocol. In brief, 100  $\mu$ l of plasma from each patient was pipetted into wells coated with an antibody specific for human PDPN. The plate was incubated for 2.5 h at room temperature to allow binding of the immobilized antibody to PDPN in the plasma. After washing the wells, biotinylated anti-human PDPN antibody was added, followed by incubation for 1 h at room temperature. After washing, bound PDPN was incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 45 min at room temperature; the binding of HRP-conjugated streptavidin was detected with 3,3',5,5'-tetramethylbenzidine substrate solution. The intensity of the color was measured at a wavelength of 450 nm.

*Immunohistochemical procedures and evaluation.* Immunohistochemistry was performed using 32 tissue samples of stages II and III from the Yamanashi University cohort. Formalin-fixed, paraffin-embedded tissue was cut into 4- $\mu$ m slices that were placed on glass slides. Slides were deparaffinized using xylene and rehydrated using a graded series of ethanol solutions. Antigen retrieval was performed by heating the samples in Dako Target Retrieval Solution (Agilent Technologies, Inc.) for 20 min at 120°C. Endogenous peroxidases were quenched using peroxidase blocking reagent (Dako; Agilent Technologies, Inc.). Sections were incubated overnight at 4°C with D2-40 monoclonal antibody (cat. no. 413451; not diluted; Nichirei Biosciences, Inc.). After washing, immunoperoxidase staining was performed using a Vectastain ABC elite kit (Vector Laboratories) and 3,3'-diaminobenzidine tablet (Fujifilm) according to the manufacturers' instructions, followed by counterstaining with hematoxylin. Lymphatic

Table I. Clinicopathological features and their relationship with plasma PDPN levels in patients with gastric cancer from the development cohort (n=46).

Variable	PDPN levels in plasma		P-value
	Low	High	
Sex			0.434
Male	10 (50.0)	16 (61.5)	
Female	10 (50.0)	10 (38.5)	
Age, years	74.6 $\pm$ 8.3	70.6 $\pm$ 12.3	0.225
Tumor size, mm	62.4 $\pm$ 39.7	65.7 $\pm$ 49.2	0.805
Depth of tumor			0.212
T2-3	16 (80.0)	16 (61.5)	
T4	4 (20.0)	10 (38.5)	
Lymph node metastasis			0.350
Negative	5 (25.0)	11 (42.3)	
Positive	15 (75.0)	15 (57.7)	
Lymphatic invasion			0.883
Negative	5 (25.0)	7 (26.9)	
Positive	15 (75.0)	19 (73.1)	
Venous invasion			0.802
Negative	4 (20.0)	6 (23.1)	
Positive	16 (80.0)	20 (76.9)	
pStage			0.208
I	1 (5.0)	4 (15.4)	
II	11 (55.0)	17 (65.4)	
III	8 (40.0)	5 (19.2)	
Lauren classification			0.079
Intestinal type	14 (70.0)	11 (42.3)	
Diffuse type	6 (30.0)	15 (57.7)	

Values are expressed as the mean  $\pm$  standard deviation or n (%). PDPN, podoplanin.

epithelium stained positive for PDPN was used as a positive control for each slide. The investigators were blinded to the clinicopathological data of the patients. PDPN expression was evaluated using high-power microscopy (magnification, x100) in five different fields. PDPN expression in the stroma surrounding the cancer cells was evaluated. Samples with the presence of immunoreactivity in >10% of stromal cells were considered positive. The CAFs at the peritumoral and tumor invasion fronts were assessed.

*Cell culture.* The human gastric cancer cell lines NUGC-3 and MKN74 were used in the present study. Cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank and were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA), 100  $\mu$ g/ml streptomycin (Sigma-Aldrich; Merck KGaA) and 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). Cells were incubated in a 5% carbon dioxide atmosphere at 37°C.

Table II. Clinicopathological features and their relationship with plasma PDPN expression in patients with gastric cancer (n=130).

Variable	PDPN expression in plasma		Univariate P-value	Multivariate		
	Low	High		Odds ratio	95% CI	P-value
Sex			0.031	1.80	0.852-3.788	0.124
Male	48 (68.6)	30 (50.0)				
Female	22 (31.4)	30 (50.0)				
Age, years	71.8±8.1	68.0±12.5	0.085			
Tumor size, mm	58.9±32.3	70.5±43.1	0.186			
Depth of tumor			0.976			
T2-3	50 (71.4)	43 (71.7)				
T4	20 (28.6)	17 (28.3)				
Nodal status			0.846			
Negative	28 (40.0)	23 (38.3)				
Positive	42 (60.0)	37 (61.7)				
Lymphatic invasion			0.926			
Negative	18 (25.7)	15 (25.0)				
Positive	52 (74.3)	45 (75.0)				
Venous invasion			0.185			
Negative	25 (35.7)	15 (25.0)				
Positive	45 (64.3)	45 (75.0)				
Lauren classification			0.002	2.73	1.308-5.703	0.008
Intestinal	48 (68.6)	25 (41.7)				
Diffuse	22 (31.4)	35 (58.3)				

Values are expressed as the mean ± standard deviation or n (%). CI, confidence interval; PDPN, podoplanin.

**Biological functional analysis of soluble PDPN in gastric cancer cells.** To assess whether soluble PDPN is able to alter the phenotype of gastric cancer cells, cells were treated with PDPN protein and then subjected to the assays specified below. As the PDPN protein, the Podoplanin/Fc Chimera, Human, Recombinant, carrier-free (R&D Systems, Inc.) was used.

**Migration and invasion assays.** A migration assay was performed using Falcon cell culture inserts with 8-μm pore membranes for use with 12-well plates (Corning, Inc.). Furthermore, an invasion assay was performed using Falcon cell culture inserts with 8-μm pore membranes for use in 24-well plates (Corning, Inc.), for which the insert was coated with Biocoat Matrigel® (BD Biosciences) prior to use.

In the migration and invasion assays, gastric cancer cells (2x10<sup>5</sup> cells/ml) were seeded in the upper chambers in FBS-free medium with or without PDPN. The medium volume in the migration assay was 1 ml and the medium volume in the invasion assay was 500 μl. PDPN protein was used at a concentration of 2 μg/ml. Medium containing 10% FBS was added to the lower chambers.

After incubation for 24 h, cells that had not migrated or invaded through the pores were removed using cotton swabs. The migrated or invaded cells were fixed and stained with Diff-Quick staining reagent. Cells were counted in four independent fields at a magnification of x100 using a BZ-X9000

All-in-One fluorescence microscope and BZ-X Analyzer Software Hybrid cell count (Keyence Corporation).

**Proliferation assay.** Gastric cancer cells were seeded into 12-well plates at a concentration of 2.0x10<sup>5</sup>/ml and then treated with PDPN at 2 μg/ml. Cells were then incubated for 48 h and subsequently peeled off and counted by the Automated cell counter Countess (Invitrogen; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Continuous variables in each group were compared using the Wilcoxon signed-rank test. The  $\chi^2$  test and Fisher's test were used to compare the categories of each group. The Cox proportional hazards model was used to investigate the clinicopathological factors that were significantly associated with plasma PDPN. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Differences were assessed using a two-sided test P<0.05 was considered to indicate a statistically significant difference.

## Results

**PDPN detection in plasma.** The range of the plasma PDPN concentration was 0-678.6 ng/ml. In the present study, the cut-off value of the plasma-PDPN concentration was set to the median plasma-PDPN concentration in the development

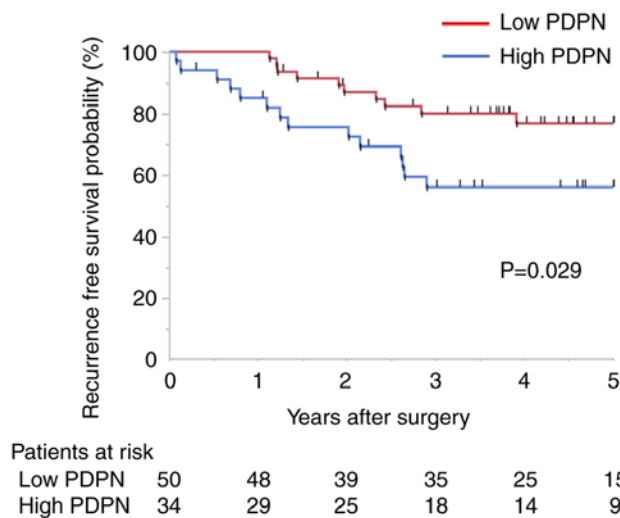


Figure 1. Kaplan-Meier curves for the recurrence-free survival rates of 84 patients with gastric cancer according to plasma PDPN levels. The recurrence-free survival rate was significantly lower in the high-PDPN group ( $P=0.029$ ). The log-rank test was used for the statistical analyses. PDPN, podoplanin.

cohort that was then divided into the high-PDPN and low-PDPN groups. The cut-off value was 0.6 ng/ml.

The associations between the clinicopathological characteristics of patients with gastric cancer and the results of the plasma PDPN status determined with PDPN ELISAs are provided in Tables I and II. In the development cohort, there were no significant differences in clinicopathological parameters between the two groups; however, the high-PDPN group tended to have a higher proportion of diffuse-type gastric cancer according to their Lauren classification (14) ( $P=0.079$ ; Table I). Multivariate logistic regression analysis of all 130 patients in both cohorts revealed that a high-PDPN status was significantly associated with diffuse-type gastric cancer ( $P=0.008$ ; Table II).

The prognostic impact of PDPN in the plasma was then investigated in the validation cohort (Fig. 1). Kaplan-Meier survival estimates indicated that the recurrence-free survival (RFS) rate was significantly lower ( $P=0.029$ ) and the overall survival rate tended to be lower ( $P=0.126$ ) in the high-PDPN group (Fig. S1). The 5-year RFS rate in the PDPN-positive group was 56.1% and that in the negative group was 76.8%. There was no significant difference in recurrence patterns between the high- and low-PDPN groups. A subgroup analysis of 36 patients with diffuse-type gastric cancer according to the levels of plasma PDPN expression revealed that the RFS rate tended to be lower in the high-PDPN group than in the low-PDPN group, but it was not significant ( $P=0.166$ ; Fig. S1).

**Association between tissue and plasma PDPN expression.** To investigate the origin of soluble plasma PDPN, the association between plasma PDPN expression and tissue PDPN expression was examined through immunohistochemical staining. Representative results of the immunohistochemical detection of the PDPN protein are provided in Fig. 2. Of the 46 patients in the development cohort, the data of 32 patients, for whom both tissue and plasma samples were obtained, were examined.

Table III. Clinicopathological characteristics of patients with Stage II/III gastric cancer with both tissue and plasma samples from the development cohort ( $n=32$ ).

Variable	Podoplanin expression in plasma		P-value
	Low	High	
Sex			0.760
Male	8 (47.1)	8 (53.3)	
Female	9 (52.9)	7 (46.7)	
Age, years	75.1 $\pm$ 8.2	69.7 $\pm$ 12.8	0.162
Tumor size, mm	68.1 $\pm$ 41.6	69.7 $\pm$ 53.4	0.925
Depth of tumor			0.062
T2,3	14 (82.4)	7 (46.7)	
T4	3 (17.7)	8 (53.3)	
Lymph node metastasis			0.243
Negative	3 (17.7)	6 (40.0)	
Positive	14 (82.4)	9 (60.0)	
Lymphatic invasion			0.736
Negative	3 (17.7)	2 (13.3)	
Positive	14 (82.4)	13 (86.7)	
Venous invasion			0.499
Negative	4 (23.5)	2 (13.3)	
Positive	13 (76.5)	13 (86.7)	
Lauren classification			0.280
Intestinal type	12 (70.6)	7 (46.7)	
Diffuse type	5 (29.4)	8 (53.3)	
IHC of tumor invasion front			0.027
Negative	14 (82.4)	6 (40.0)	
Positive	3 (17.6)	9 (60.0)	

Values are expressed as the mean  $\pm$  standard deviation or n (%). IHC, immunohistochemistry.

Among these, 15 patients had high plasma PDPN expression. PDPN expression on the tumor invasion front was observed in 12 patients, of whom 9 (75%) had high plasma PDPN expression. By contrast, 14 (70%) of the 20 patients who did not exhibit PDPN expression on the tumor invasion front had low plasma PDPN expression ( $P=0.027$ ; Table III). These results indicated that plasma PDPN may reflect PDPN expression in CAFs at the tumor invasion front.

**Biological analysis of soluble PDPN.** To investigate whether soluble PDPN itself is able to affect phenotypic changes in gastric cancer cells, NUGC-3 and MKN74 cells were treated with soluble PDPN.

In the migration and invasion assays, the addition of PDPN did not enhance the migratory ability compared with the control. In addition, there was no significant difference in proliferative ability between the two groups (Figs. S2 and S3).



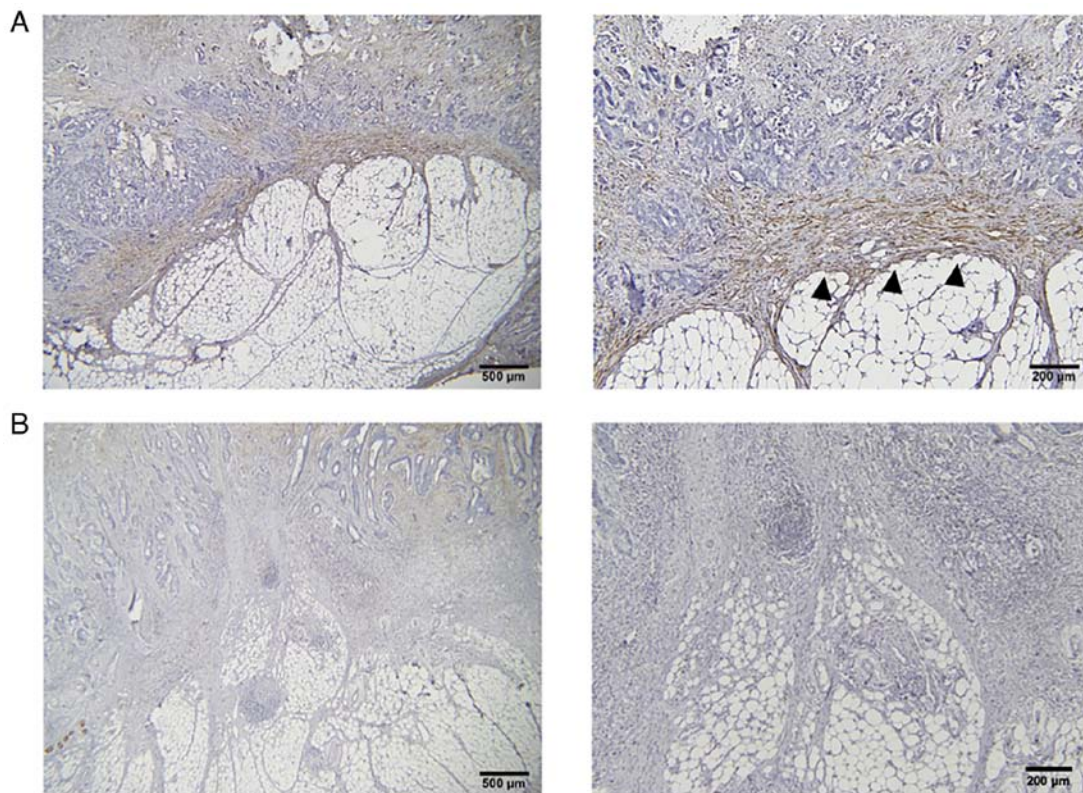


Figure 2. Representative results of the immunohistochemical detection of PDPN protein in gastric cancer. PDPN is not expressed in gastric cancer cells but in stromal cells (scale bar, 500 and 200  $\mu\text{m}$ ). (A) PDPN is expressed in CAFs at the tumor invasion front (arrowheads). (B) PDPN is not expressed in CAFs at the tumor invasion front. PDPN, podoplanin; CAF, cancer-associated fibroblast.

## Discussion

Diffuse-type gastric cancer, also known as scirrhous gastric cancer, has high invasion and metastatic potential, causes peritoneal metastasis at an early stage and is considered to have a poor prognosis despite various treatment strategies (15). Diffuse-type gastric cancer is characterized by a larger number of stromal cells, called CAFs, than the number of cancer cells in the tissue (16). The present study focused on CAFs in gastric cancer and examined their potential as a blood biomarker. Identification of PDPN, one of the representative CAF markers in gastric cancer, in the plasma was significantly correlated with diffuse-type gastric cancer and patients with gastric cancer with high plasma PDPN expression had poor prognosis.

Various molecules, such as  $\alpha$ -SMA, FAP and PDPN, have been reported as CAF markers for gastric cancer (9,10). A previous study by our group reported that PDPN is a CAF marker for gastric cancer and demonstrated the prognostic impact of PDPN expression in patients with gastric cancer (11). Liu *et al* (17) also demonstrated that high PDPN expression in tissues reduces the sensitivity to adjuvant chemotherapy and correlates with poor prognosis of patients with gastric cancer. PDPN is a mucin-like transmembrane glycoprotein that is widely used as a marker for the lymphatic endothelium (18). PDPN expression in cancer cells has been reported in various tumor types, including brain tumors, esophageal squamous cell carcinoma, angiosarcoma, mesothelioma, squamous cell carcinoma of the lung and cervical carcinoma (19,20). By

contrast, in gastric and colorectal cancers, PDPN expression is low in cancer cells but high in CAFs (11,21).

In the present study, the association between plasma PDPN expression and tissue PDPN expression was examined to investigate the origin of soluble plasma PDPN. Plasma PDPN expression was associated with PDPN expression at the tumor invasion front, suggesting that plasma PDPN may reflect the number of CAFs at the tumor invasion front of the tissue and may be a surrogate marker for CAFs in patients with gastric cancer. Neri *et al* (22) reported that PDPN-positive CAFs at the tumor invasion front lead and enhance the local invasion of cancer cells *in vitro*. Thus, plasma PDPN may be an indication of CAF activity, which promotes tumor progression.

Zhao *et al* (23) demonstrated that the plasma levels of soluble PDPN reflect tumor dynamics prior to and after treatment in patients with gastric cancer. In the present study, it was also demonstrated that soluble PDPN not only reflects the amount of stroma in patients with gastric cancer but also has potential as a prognostic biomarker. Kemi *et al* (24) reported that the amount of stroma in gastric cancer tissues correlates with poor prognosis in patients with gastric cancer. Furthermore, the present results not only demonstrated that plasma PDPN expression may help determine the prognosis of patients with diffuse-type gastric cancer but indicated that patients with intestinal-type gastric cancer with high plasma PDPN expression may also tend to have unfavorable prognosis, although there was no significant influence (data not shown). These results suggest the possibility that PDPN-based liquid biopsy has the potential to detect hidden diffuse-type gastric

cancers. The plasma-based PDPN liquid biopsy performed in the present study may not only reflect the amount of stroma in patients with gastric cancer but may also enable comprehensive assessment of a patient's status without being affected by intra-tissue heterogeneity.

It remains elusive whether the soluble PDPN examined in the present study is a functional secretory protein or CAF-derived membrane debris. In the molecular biological analysis, no phenotypic changes were observed in cancer cells due to the direct administration of soluble PDPN. However, the mechanisms underlying platelet-mediated cancer progression may also be considered. Suzuki-Inoue *et al* (25) reported that PDPN is an *in vivo* ligand of the platelet activation receptor CLEC-2. Platelets have a major role in cancer progression by adhering to cancer cells and supporting metastasis (26). Soluble PDPN may indirectly affect cancer progression via platelet activation. In addition, cell-cell communication through microvesicles may be considered. Carrasco-Ramírez *et al* (27) reported that PDPN protein was transmitted between cells as a component of extracellular vesicles. It may be necessary to examine the functional changes of gastric cancer cells by cell-cell communication of PDPN using extracellular vesicles instead of direct administration of PDPN protein.

The present study has certain limitations. The study had a small sample size and a retrospective design; therefore, bias was likely to be present and influence the clinicopathological findings. Furthermore, since the observation period in the development cohort was short and it was not possible to analyze the survival data, the survival outcome was analyzed in a small sample using the validation cohort; thus, it was not possible to draw any concrete conclusions regarding the prognostic impact of a plasma-based PDPN liquid biopsy.

In conclusion, the present study focused on PDPN, a representative marker of CAFs in gastric cancer, and investigated its potential as a blood biomarker. Plasma soluble PDPN was indicated to be a surrogate marker for diffuse-type gastric cancer and may reflect the prognosis of gastric cancer.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Authors' contributions

Conceptualization: KS and DI; Methodology: KoT, KS, TN, KaT, RS, AY, SF; Formal analysis and investigation: KoT, KS, HidenoA, NH, YK, HidetA, HiromK, SI, HirosK, HiroTK and EO; Writing - original draft preparation: KoT; Writing - review and editing: KS, DI and EO. KS and KoT

checked and approved the authenticity of the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the ethics committee of the University of Yamanashi Hospital (Yamanashi, Japan; approval no. 2301) and Kyoto Prefectural University of Medicine (Kyoto, Japan) and was performed in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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