

# Clinical significance of platelet-derived growth factor receptor- $\beta$ gene expression in stage II/III gastric cancer with S-1 adjuvant chemotherapy

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**Abstract.** Overall survival remains unsatisfactory in stage II/III gastric cancer, even after curative surgery and adjuvant chemotherapy. Platelet-derived growth factor receptor- $\beta$  (*PDGFR- $\beta$* ) is associated with the proliferation of cancer cells. The present study therefore investigated the association of *PDGFR- $\beta$*  gene expression with patient outcome in 134 stage II/III gastric cancer patients who received adjuvant chemotherapy with S-1. Relative *PDGFR- $\beta$*  gene expression was measured in surgical cancer tissue and adjacent normal mucosa specimens by reverse transcription-quantitative polymerase chain reaction. The *PDGFR- $\beta$*  gene expression levels were found to be significantly higher in the cancer tissues compared with the adjacent normal mucosa. A high level of *PDGFR- $\beta$*  gene expression was associated with a significantly poorer 5-year overall survival rate compared with a low level of *PDGFR- $\beta$*  expression. Upon multivariate analysis, *PDGFR- $\beta$*  gene expression was found to be an independent predictor of survival. Overall, the study indicates that *PDGFR- $\beta$*  overexpression in gastric cancer tissues is a useful independent predictor of outcome in patients with stage II/III gastric cancer who receive adjuvant chemotherapy with S-1.

## Introduction

Gastric cancer is the fourth most common cancer globally and the second leading cause of cancer-associated mortality (1).

Gastrectomy with D2 dissection and progress in chemotherapy have significantly improved survival times in patients with gastric cancer (2,3). The standard treatment for stage II/III gastric cancer is curative resection and adjuvant chemotherapy. On the basis of the results of the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer, patients with stage II/III gastric cancer in Japan usually receive S-1 for 1 year after gastrectomy. However, despite treatment with S-1, the 5-year overall survival rate is 71.7% and thus remains unsatisfactory (4). Therefore, great hope has been placed on the development of personalized medicine using biomarkers to improve outcomes in stage II/III gastric cancer.

Platelet-derived growth factor receptor- $\beta$  (*PDGFR- $\beta$* ) is a cell surface tyrosine kinase receptor for members of the *PDGF* family. *PDGFR- $\beta$*  is expressed in numerous types of human neoplasms, including gastric cancer (5-7). *PDGFR- $\beta$*  signaling has been reported to increase tumor cell proliferation in an autocrine manner (8), and to stimulate angiogenesis (9), recruit pericytes (8,10) and regulate interstitial fluid pressure (IFP) in the stroma; *PDGFR- $\beta$*  thereby affects the transvascular transport of chemotherapeutic agents in a paracrine manner (11).

The present study was designed to evaluate the clinical significance of *PDGFR- $\beta$*  gene expression in patients with stage II/III gastric cancer who received curative resection followed by adjuvant chemotherapy with S-1.

## Materials and methods

**Patients and samples.** The present study focused on surgically resected specimens of cancer tissue and adjacent normal mucosa obtained from 134 patients with stage II/III gastric cancer who underwent curative surgery without receiving pre-operative chemotherapy or radiotherapy. Tumor stage was evaluated according to the 7th edition of the International Union Against Cancer (UICC)-TNM classification of malignant tumors (12). The patients post-operatively received adjuvant chemotherapy with S-1. All patients were treated in the Department of Surgery, Yokohama City University (Yokohama,

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**Key words:** *PDGFR- $\beta$* , tyrosine kinase receptor, stage II/III gastric cancer, adjuvant chemotherapy with S-1, prognostic factor

Kanagawa, Japan), the Gastroenterological Center, Yokohama City Medical Center (Yokohama, Kanagawa, Japan) or the Department of Gastrointestinal Surgery, Kanagawa Cancer Center (Yokohama, Kanagawa, Japan) between March 2002 and July 2010. Informed consent was obtained from all patients, and the study protocol was approved by the Ethics Committees of Yokohama City University Medical Center, Yokohama City University and Kanagawa Cancer Center. All tissue samples were embedded in optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and immediately stored at  $-80^{\circ}\text{C}$  until use. No patient had any other malignancies. The tissue specimens were stained with hematoxylin and eosin, and examined histopathologically. Sections that consisted of  $>80\%$  carcinoma cells were used to prepare total RNA.

**RNA extraction and complementary DNA (cDNA) synthesis.** Total RNA isolated from gastric cancer tissues and adjacent normal mucosa was prepared using TRIzol (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized from  $0.4\text{ }\mu\text{g}$  total RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's instructions. Following synthesis, the cDNA was diluted to  $0.2\text{ }\mu\text{g}/\mu\text{l}$  with water and stored at  $-20^{\circ}\text{C}$  until use.

**Oligonucleotide primers for PDGFR- $\beta$  cDNA amplification by reverse-transcription polymerase chain reaction (RT-PCR).** The oligonucleotide primers for PDGFR- $\beta$  were as follows: Sense, 5'-CGTGGCTTTTCTGGTATCTTTGAG-3' and antisense, 5'-CGTTGATGGATGACACCTGGAG-3'.  $\beta$ -actin was used as the internal control. The oligonucleotide primers for  $\beta$ -actin were as follows: Sense, 5'-AGTTGCGTTACACCC TTTCTTGAC-3' and antisense, 5'-GCTCGCTCCAACCGA CTGC-3'.

Amplification of PDGFR- $\beta$  was performed for 40 cycles of 10 sec at  $95^{\circ}\text{C}$ , 10 sec at  $58^{\circ}\text{C}$  and 20 sec at  $72^{\circ}\text{C}$ . Amplification of  $\beta$ -actin was performed for 40 cycles of 15 sec at  $95^{\circ}\text{C}$ , 15 sec at  $60^{\circ}\text{C}$  and 30 sec at  $72^{\circ}\text{C}$ . A  $10\text{-}\mu\text{l}$  aliquot of each amplified PCR product underwent electrophoresis in a 3% agarose gel containing ethidium bromide.

**Immunohistochemical staining.** Immunohistochemical studies of PDGFR- $\beta$  were performed on formalin-fixed, paraffin-embedded surgical specimens obtained from the gastric cancer patients. The section width was specified as  $4\text{ }\mu\text{m}$ . The tissue sections were deparaffinized and soaked in 10 mM sodium citrate buffer (pH 6.0) at  $121^{\circ}\text{C}$  for 15 min to retrieve cell antigens. Blocking was performed twice. First, endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution for 5 min. Next, protein activity was blocked with 5% skimmed milk for 5 min. Subsequent to blocking, the sections were incubated overnight at  $4^{\circ}\text{C}$  to allow antigen-antibody reactions to occur. Peroxidase-labeled polymer (EnVision+, rabbit; Dako, Glostrup, Denmark) was used to detect signals of the antigen-antibody reactions. All sections were counterstained with hematoxylin. The primary rabbit polyclonal antibodies against PDGFR- $\beta$  (catalog no., HPA028499; Atlas Antibodies, Stockholm, Sweden) were used at a dilution of 1:100.

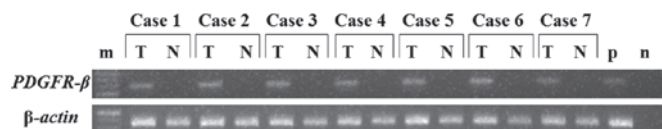


Figure 1. Reverse-transcription polymerase chain reaction. Expression of platelet-derived growth factor receptor- $\beta$  (PDGFR- $\beta$ ) mRNA was significantly higher in the cancer tissue (T) than in the paired normal adjacent mucosa (N). m, (molecular) marker; p, positive control; n, negative control.

**RT-quantitative (q)PCR.** RT-qPCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.). PCR was performed in a total volume of  $15\text{ }\mu\text{l}$ , which included  $0.2\text{ }\mu\text{g}$  cDNA,  $0.4\text{ }\mu\text{M}$  of each primer,  $7.5\text{ }\mu\text{l}$  iQ SYBR-Green Supermix containing dATP, dCTP, dGTP and dTTP at concentrations of  $400\text{ }\mu\text{M}$  each, and 50 U/ml of iTaq DNA polymerase. The PCR cycling conditions consisted of 10 min at  $95^{\circ}\text{C}$ , followed by 40 cycles of denaturation of the cDNA for 10 sec at  $95^{\circ}\text{C}$ , annealing for 10 sec at  $56^{\circ}\text{C}$  ( $60^{\circ}\text{C}$  for  $\beta$ -actin) and a primer extension for 20 sec at  $72^{\circ}\text{C}$ , followed by 10 min at  $72^{\circ}\text{C}$ . To distinguish specific from non-specific products and primer dimers, melting curve analyses were performed. To evaluate specific mRNA expression in the samples, a standard curve was created for each run, based on three points from human control cDNA (Clontech Laboratories, Inc., CA, USA). The concentrations of each sample were calculated by relating their crossing point to the standard curve. The number of experimental repeats was three times, and the method used for quantitation was relative quantities (iQ5 software version 2.0; Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Differences between gene expression levels in the gastric cancer and adjacent normal mucosa samples were compared using the Wilcoxon test. The associations between gene expression and potential explanatory variables, including age, gender, tumor size, histological type, serosal invasion, lymph node metastasis, tumor-node-metastasis (TNM) stage (12), lymphatic invasion and venous invasion, were evaluated using the  $\chi^2$  test. Associations between PDGFR- $\beta$  gene expression and survival were assessed using the Kaplan-Meier method and were compared by the log-rank test. A Cox proportional-hazards model was used to perform univariate analyses and stepwise multivariate analyses to determine risk factors. All statistical analyses were performed with the Dr. SPSS II program, version 11.0.1 J for Windows (SPSS, Inc., Chicago, IL, USA). Two-sided P-values were calculated, and a difference was considered statistically significant at  $P < 0.05$ . Data are expressed as median (range).

## Results

**PDGFR- $\beta$  mRNA expression in specimens of cancer tissue and adjacent normal mucosa.** PDGFR- $\beta$  gene expression was examined by RT-PCR, using gastric cancer tissue and normal gastric mucosal tissue obtained from 7 patients (Fig. 1). The results revealed that PDGFR- $\beta$  expression was significantly higher in tumor tissue than in normal gastric mucosal tissue. Therefore, PDGFR- $\beta$  gene expression was examined by RT-qPCR in gastric cancer tissue and normal gastric mucosa

Table I. Association between *PDGFR-β* mRNA expression and clinicopathological features.

Variable	Low <i>PDGFR-β</i> expression (n=67)	High <i>PDGFR-β</i> expression (n=67)	P-value
Age, years			
<65	26	30	0.484
≥65	41	37	
Gender			
Male	45	47	0.710
Female	22	20	
Tumor size, cm			
<6	27	33	0.297
≥6	40	34	
Histological type			
Differentiated	24	29	0.377
Undifferentiated	43	38	
Serosal invasion			
Absent	27	26	0.860
Present	40	41	
Lymph node metastasis			
Absent	8	8	1.000
Present	59	59	
TNM stage			
II	19	21	0.706
III	48	46	
Lymphatic invasion			
Absent	14	16	0.679
Present	53	51	
Venous invasion			
Absent	21	12	0.071
Present	46	55	

*PDGFR-β*, platelet-derived growth factor receptor-β; TNM, tumor-node-metastasis.

in 75 patients for whom samples of both gastric cancer tissue and normal gastric mucosal tissue were available. The mRNA expression levels of *PDGFR-β* in specimens of cancer tissue and adjacent normal mucosa obtained from 7 patients with gastric cancer were analyzed by RT-PCR (Fig. 1). *PDGFR-β* mRNA expression was significantly higher in the cancer tissues than in the paired normal adjacent mucosa. *PDGFR-β* mRNA expression was confirmed in clinical samples (n=75) by RT-qPCR. Expression levels of the *PDGFR-β* gene were significantly higher in the cancer tissues compared with the adjacent normal mucosa (P=0.009; Fig. 2).

**Immunohistochemistry of *PDGFR-β* expression.** Expression of *PDGFR-β* protein was evaluated by immunohistochemical analysis of resected specimens of gastric cancer. Positive staining for *PDGFR-β* was observed in the tumor stromal cells of the gastric cancer tissue in differentiated and undifferentiated types of gastric cancer (Fig. 3).

**Associations between *PDGFR-β* mRNA expression levels and clinicopathological features.** Expression levels of the

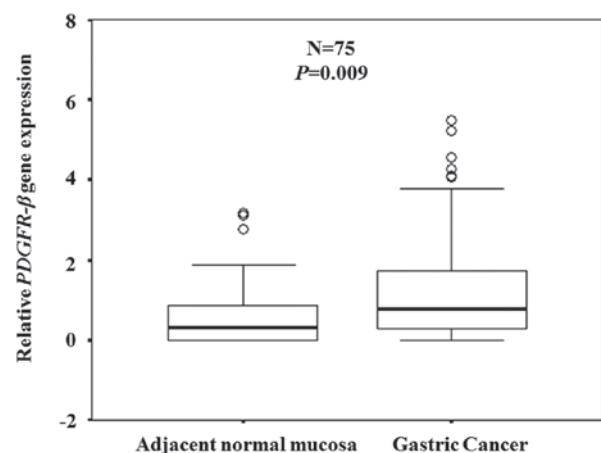
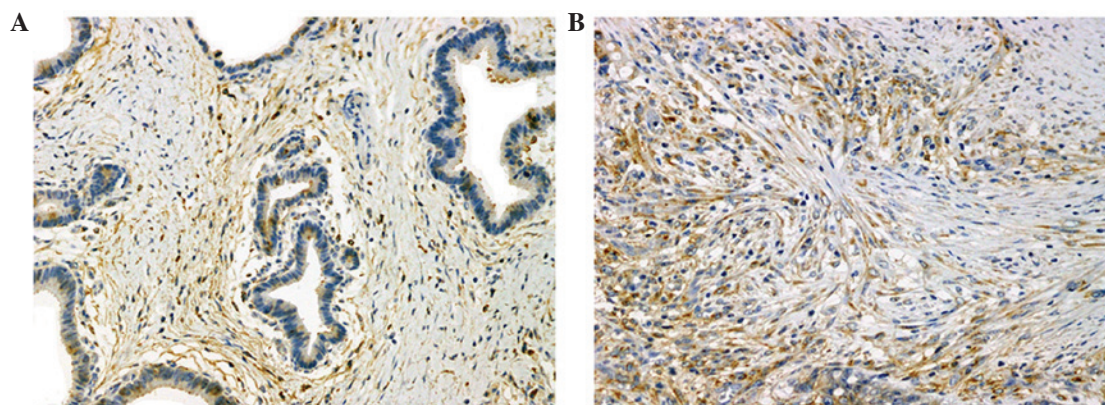


Figure 2. Comparison of platelet-derived growth factor receptor-β (*PDGFR-β*) mRNA expression levels between gastric cancer tissue and adjacent normal mucosa. Box boundaries represent the 25th and 75th percentiles of the observed values, capped bars represent the 10th and 90th percentiles, and the solid line represents the median. P-values were calculated by the Wilcoxon signed-rank test. Expression levels of the *PDGFR-β* gene were higher in the cancer tissues than in the adjacent normal mucosa (P=0.009).

Table II. Univariate and multivariate Cox proportional hazards analysis of clinicopathological factors.

Variable	n	Univariate			Multivariate		
		Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age, years							
<65	56	1	0.356-1.319	0.258			
≥65	78	0.685					
Gender							
Male	92	1	0.318-1.438	0.310			
Female	42	0.676					
Tumor size, cm							
<6	60	1	0.553-2.060	0.847			
≥6	74	1.067					
Histological type							
Differentiated	53	1	0.497-1.907	0.937			
Undifferentiated	81	0.973					
Serosal invasion							
Absent	53	1	0.753-3.118	0.239			
Present	81	1.532					
Lymph node metastasis							
Absent	16	1	0.655-11.356	0.168			
Present	118	2.726					
TNM stage							
II	40	1	1.039-6.019	0.041 <sup>a</sup>	1	1.071-6.220	0.035 <sup>a</sup>
III	94	2.501			2.581		
Lymphatic invasion							
Absent	30	1	0.456-2.201	0.997			
Present	104	1.002					
Venous invasion							
Absent	33	1	0.685-3.580	0.288			
Present	101	1.566					
<i>PDGFR-β</i> expression							
Low	67	1	1.002-3.887	0.049 <sup>a</sup>	1	1.032-4.029	0.040 <sup>a</sup>
High	67	1.973			2.039		

<sup>a</sup>P<0.05. *PDGFR-β*, platelet-derived growth factor receptor-β; TNM, tumor-node-metastasis.Figure 3. Immunohistochemical analysis of platelet-derived growth factor receptor-β (*PDGFR-β*) expression. Expression of *PDGFR-β* protein was evaluated by immunohistochemical analysis of resected specimens of gastric cancer. Positive staining for *PDGFR-β* was observed in the tumor stromal cells of gastric cancer tissue in (A) differentiated and (B) undifferentiated types of gastric cancer.



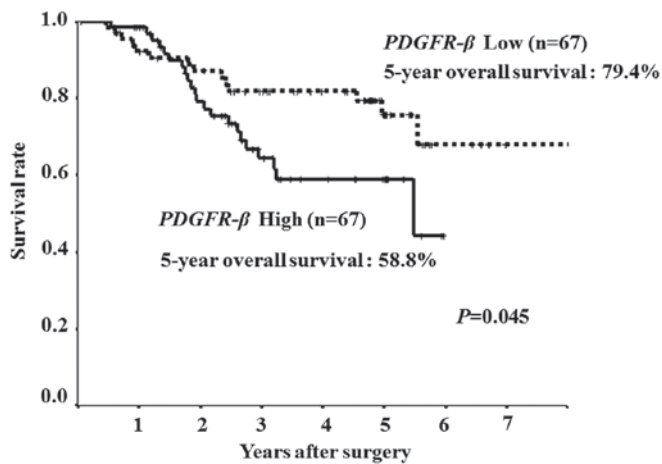


Figure 4. Association between platelet-derived growth factor receptor- $\beta$  (*PDGFR- $\beta$* ) mRNA expression and post-operative survival. Expression levels of the *PDGFR- $\beta$*  gene were categorized as low or high according to the median value. The post-operative survival rate was analyzed by the Kaplan-Meier method, and differences in survival rates were assessed by the log-rank test. Overall survival was significantly poorer in patients with high expression levels of the *PDGFR- $\beta$*  gene than in those with low expression levels ( $P=0.045$ ).

*PDGFR- $\beta$*  gene were categorized as low or high according to the median value. The associations between gene expression and clinicopathological features were then examined. Expression levels of the *PDGFR- $\beta$*  gene were not found to be associated with any clinicopathological variable (Table I).

**Associations between *PDGFR- $\beta$*  mRNA expression levels and outcome.** The expression levels of the *PDGFR- $\beta$*  gene were categorized as low or high according to the median expression value. Overall survival was significantly poorer in the patients with high expression levels (5-year overall survival rate, 58.8%) of the *PDGFR- $\beta$*  gene than in those with low expression levels (5-year overall survival rate, 79.4%) ( $P=0.045$ ; Fig. 4).

**Univariate and multivariate analyses of the associations between clinicopathological factors and *PDGFR- $\beta$*  mRNA expression levels with regard to outcome.** The following variables were included in univariate analysis: Age, gender, tumor size, histological grade, serosal invasion, lymph-node metastasis, TNM stage, lymphatic invasion, venous invasion and *PDGFR- $\beta$*  gene expression. Only TNM stage and *PDGFR- $\beta$*  gene expression were significantly associated with the outcome. Upon multivariate Cox proportional-hazards regression analysis, TNM stage ( $P=0.035$ ) and high *PDGFR- $\beta$*  gene expression ( $P=0.040$ ) was independently and inversely associated with outcome (Table II).

## Discussion

The standard treatment for stage II/III gastric cancer is a curative resection and adjuvant chemotherapy. The overall survival rate of patients with stage II/III gastric cancer remains unsatisfactory, even after curative surgery and adjuvant chemotherapy with S-1 (2-4). Improved risk stratification and personalized medicine based on biomarker analysis will hopefully improve outcomes in stage II/III gastric cancer. The

present study therefore focused on *PDGFR- $\beta$*  and examined the clinical significance of *PDGFR- $\beta$*  expression in patients with stage II/III gastric cancer who received curative surgery and post-operative adjuvant chemotherapy with S-1.

The present study examined the expression levels of *PDGFR- $\beta$*  mRNA in gastric cancer and adjacent normal mucosa. Studies have previously compared the relative mRNA expression levels of the *PDGFR- $\beta$*  gene between various types of cancer tissue and adjacent normal mucosa. Erben *et al* reported that *PDGFR- $\beta$*  mRNA expression is higher in rectal cancer tissue than in normal rectal mucosa (13). Antoniadis *et al* found that *PDGF* and *PDGFR- $\beta$*  mRNA are strongly expressed in lung carcinoma tissue, but not in normal lung tissue (14). Vrekoussis *et al* showed that immunohistochemical expression levels of endothelial *PDGFR- $\beta$*  are significantly higher in breast cancer tissue than in normal breast tissue (15), and Guo *et al* reported that the positive rate of immunohistochemical *PDGFR- $\beta$*  expression is significantly higher in gastric carcinoma tissue than in normal gastric mucosa tissue (16). The present results are consistent with these findings. *PDGFR- $\beta$*  mRNA expression levels were significantly higher in 75 specimens of gastric cancer tissue than in the paired adjacent normal mucosa.

The associations between *PDGFR- $\beta$*  mRNA expression levels and clinicopathological features in gastric cancer were then evaluated. Kodama *et al* reported that *PDGFR- $\beta$*  expression correlates with lymph node metastasis in gastric cancer (5). Suzuki *et al* showed that activation of *PDGFR- $\beta$*  correlates with the depth of tumor invasion in gastric cancer (17), and Guo *et al* reported that *PDGFR- $\beta$*  expression positively correlates with the depth of tumor invasion, lymph node metastasis and TNM stage in gastric cancer (16). By contrast, the expression levels of *PDGFR- $\beta$*  mRNA were not associated with any clinicopathological features in the present study.

Finally, the present study assessed the associations between *PDGFR- $\beta$*  gene expression levels and outcome in stage II/III gastric cancer after curative resection and adjuvant chemotherapy with S-1. Paulsson *et al* reported that high stromal *PDGFR- $\beta$*  expression significantly correlates with shorter recurrence-free and cancer-specific survival times in various types of breast cancer (18), while Häggblöf *et al* showed that stromal *PDGFR- $\beta$*  expression predicts cancer-specific survival in various types of prostate cancer (19). Sato *et al* (20) reported that *PDGFR- $\beta$*  expression is a significant prognostic factor in grade II/III astrocytoma and grade IV glioblastoma. Chen *et al* (21) found that *PDGFR- $\beta$*  overexpression alone is not a significant predictor of either disease-free survival or overall survival, whereas *PDGFR- $\alpha$* /*PDGFR- $\beta$* /vascular endothelial growth factor coexpression significantly correlates with poorer disease-free survival and overall survival in patients with stage I-IV hepatocellular carcinoma. By contrast, Shinohara *et al* reported that the levels of *PDGFR- $\beta$*  expression are not a statistically significant predictor of 5-year overall survival in either limited or extensive small-cell lung cancer (22). The present results showed that high *PDGFR- $\beta$*  expression levels were a significant independent predictor of a poorer 5-year overall survival rate in patients with stage II/III gastric cancer after curative resection and post-operative adjuvant chemotherapy with S-1. Upon multivariate Cox proportional hazards

regression analysis, high levels of *PDGFR- $\beta$*  gene expression were independently and inversely associated with outcome.

The molecular mechanisms and functional impact of *PDGFR- $\beta$*  expression in cancer remain to be fully elucidated. *PDGFR- $\beta$*  promotes the proliferation of tumor-associated fibroblasts and neovascular endothelial cells participating in tumor angiogenesis; it also increases IFP, and a higher IFP is associated with greater *PDGFR- $\beta$*  expression. Tailor *et al* reported that the IFP was higher in the high *PDGFR- $\beta$*  group in nude mice bearing human non-small-cell lung cancer A549 xenografts (23). Increased IFP acts as a barrier to drug distribution and the migration of cytotoxic T lymphocytes that target tumor cells (24). High IFP is thus a prognostic factor of tumors. Yeo *et al* reported that IFP is a prognostic factor in patients with cervical cancer (25). Milosevic *et al* (26) also showed that IFP is a predictor of survival in patients with cervical cancer. These findings suggest that reducing the IFP may contribute to improved survival. Pietras *et al* (27) demonstrated that the inhibition of *PDGFR* reduces the IFP and increases the capillary-to-interstitium transport of  $^{51}\text{Cr}$ -EDTA in PROb rat colonic carcinomas. Pietras *et al* (28) also reported that the inhibition of *PDGFR* signaling enhances the antitumor effect of chemotherapy on subcutaneous KAT-4 tumors in FOX Chase severe combined immune deficiency mice. Emerich *et al* (29) showed that infusion of the bradykinin agonist Cereport (labradimil or RMP-7) in rats bearing experimental tumors reduces the IFP and increases deposition of  $^{14}\text{C}$ -carboplatin in tumor tissue. These findings suggest that high *PDGFR- $\beta$*  expression is associated with high IFP and causes resistance to chemotherapy by reducing blood flow to tumors and decreasing drug delivery in patients with gastric cancer after curative surgery and post-operative adjuvant chemotherapy with S-1. Imatinib is a *PDGFR* inhibitor. Wiig *et al* (30) reported that rats with colonic carcinomas treated with imatinib exhibited reduced IFP. Kim *et al* (24) demonstrated that treatment with imatinib markedly enhances the chemotherapeutic effects of antitumor drugs such as 5-fluorouracil and paclitaxel *in vivo* in gastric carcinoma MKN-45 cells transplanted into nude mice. These findings indirectly suggest that *PDGFR- $\beta$*  expression may antagonize the antitumor effects of chemotherapy. However, further investigations are necessary to elucidate the underlying mechanisms.

In conclusion, *PDGFR- $\beta$*  gene expression levels were higher in gastric cancer tissue than in adjacent normal mucosa in the present study, and the high expression of this gene was significantly associated with a poor outcome in patients with stage II/III gastric cancer who underwent curative resection and received adjuvant chemotherapy with S-1. These findings suggest that *PDGFR- $\beta$*  overexpression is a useful, independent predictor of outcome in patients with stage II/III gastric cancer who receive curative surgery and post-operative adjuvant chemotherapy with S-1.

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