Phosphatidylserine-specific Phospholipase A1 (PS-PLA$_1$) Expression in Colorectal Cancer Correlates with Tumor Invasion and Hematogenous Metastasis

YUUKI IIDA[1], EIJI SUNAMI[1], HIROHARU YAMASHITA[2], MASAYA HIYOSHI[1], SOICHIRO ISHIHARA[1], HIRONORI YAMAGUCHI[1], ASUKA INOUE[3], KUMIKO MAKIDE[3], NELSON H. TSUNO[4], JUNKEN AOKI[3], JOJI KITAYAMA[1] and TOSHIAKI WATANABE[1]

Departments of [1]Surgical Oncology, [2]Gastrointestinal Surgery, and [4]Transfusion Medicine, Faculty of Medical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan; [3]Laboratory of Molecular and Cellular Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-ku, Sendai, Miyagi, Japan

Abstract. *Background: The function of phosphatidylserine-specific phospholipase A1 (PS-PLA$_1$), a phospholipase that acts specifically on phosphatidylserine and produces lysophosphatidylserine, a lysophospholipid mediator, has not been fully elucidated. We evaluated the role of PS-PLA$_1$ in oncogenesis and metastasis of colorectal cancer (CRC). Materials and Methods: Specimens from 85 patients with CRC were immunostained with a monoclonal antibody against PS-PLA$_1$. The correlation between PS-PLA$_1$ expression and the clinicopathological variables was analyzed. Results: Tumor depth and hematogenous metastasis independently positively correlated with PS-PLA$_1$ expression. High PS-PLA$_1$ expression was associated with shorter disease-free survival, although it was not an independent predictive factor. Conclusion: PS-PLA$_1$ expression in CRC is associated with tumor invasion and metastasis.*

Phosphatidylserine-specific phospholipase A1 (PS-PLA$_1$) is a phospholipase that hydrolyzes phosphatidylserine (PS) and produces lysophosphatidylserine (LysoPS) (1). Only few reports exist in the literature about the physiological functions of LysoPS and PS-PLA$_1$. The highlighted function of LysoPS and PS-PLA$_1$ is the stimulation of histamine release from rat peritoneal mast cells, which indicates that LysoPS may be involved in allergic conditions (2). PS-PLA$_1$ or LysoPS are also involved in growth suppression of T-cells (3) and potentiation of nerve growth factor-induced neural cell differentiation (4). On the other hand, studies on the chemotactic migration of glioma cell lines and metastasis of melanoma cell lines indicate a correlation between PS-PLA$_1$ expression and tumor cell migration or tumor metastasis (5, 6). Recently, we also reported the stimulation of chemotactic migration of colorectal cancer (CRC) cell lines (7).

Since there are no reports to demonstrate the expression of PS-PLA$_1$ in human tumor tissue, we aimed to clarify the correlation between PS-PLA$_1$ expression and clinicopathological features of CRC, which is the second most commonly diagnosed cancer in females and the third in males (8). For this purpose, human CRC tissue specimens were immunostained with a monoclonal antibody against PS-PLA$_1$, and the correlation between its expression and the clinicopathological features of patients were evaluated.

Materials and Methods

Samples. A total of 85 consecutive patients with CRC who underwent surgery at the University of Tokyo hospital in the period between January 2005 and December 2005 were included. Among them, 75 patients had undergone curative surgery with lymph node dissection. Those patients diagnosed as having ulcerative colitis, familial adenomatous polyposis, or multiple CRCs, and those who underwent emergency operation, neoadjuvant chemoradiotherapy, or endoscopic mucosal resection before operation were excluded. Informed consent was obtained from all participants. Clinicopathological features of patients were obtained from their records and were analyzed based on the TNM Classification of Malignant Tumors of the Union for International Cancer Control (UICC, 7th edition) (9). Surgically-obtained specimens were preserved in 10% buffered formalin and embedded in paraffin blocks.

Correspondence to: Yuuki Iida, Department of Surgical Oncology, Faculty of Medical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel: +81 338155411, Fax: +81 338116822, e-mail: yiida-tky@umin.ac.jp

Key Words: Colorectal cancer, immunohistochemistry, phosphatidylserine-specific phospholipase A$_1$, tumor invasion, metastasis.

Table I. *Patients' characteristics*.

Variable	Number (%)		
Total number		85	
Mean age (years)±SD		64.9±11.0	
Gender	Male	51	(60.0)
	Female	34	(40.0)
Tumor location	Cecum	5	(5.9)
	Ascending colon	18	(21.2)
	Transverse colon	6	(7.1)
	Descending colon	3	(3.5)
	Sigmoid colon	24	(28.2)
	Rectum	29	(34.1)

SD: Standard deviation.

Immunostaining of PS-PLA$_1$. Rat monoclonal antibody against human PS-PLA$_1$ (6F8) was generated as described previously (10). Paraffin-embedded sections (3 μm-thick) were heated in a microwave oven for 15 min in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval and then placed into methanol with 1% hydrogen peroxide for 30 min at room temperature to suppress endogenous peroxidase activity. After blocking nonspecific reactions with rabbit serum, the sections were incubated with antibody to PS-PLA$_1$ (6F8, 1:150 dilution) overnight at 4˚C. For the color reaction, sections were incubated with labeled polymer (Histofine®, Simple Stain MAX-PO(Rat), NICHIREI BIOSCIENCES INC, Chuo-ku, Tokyo, Japan) for 30 min at room temperature, followed by treatment with a diaminobenzidine solution for 3.5 min. The sections were then counterstained with hematoxylin and analyzed.

PS-PLA$_1$ staining was mainly observed in the cytoplasm of the cancer cells, which were generally more intensely stained than the normal epithelium or stromal cells. The immunostaining intensity was classified into four grades, as follows: negative; weak; moderate; and strong (Figure 1). Sections with more than 50% of cells scored as moderate or strong were classified as PS-PLA$_1$-high, and those with 50% or less were classified as PS-PLA$_1$-low. Immunostaining of each section was assessed by two independent observers (YI and MH), without any knowledge of the patient data, and the inter-observer correlation coefficient was 0.69.

Statistical analysis. Chi-square test, Fisher's exact test or non-paired Student's *t*-test and logistic regression model were used for analysis of the correlation between PS-PLA$_1$ expression and the clinicopathological variables. Disease-free survival (DFS) was analyzed by the Kaplan-Meier method, log-rank test, and Cox proportional hazard model. Two sided *p*-values less than 0.05 were regarded as statistically significant.

Results

The clinicopathological features of the patients are shown in Table I. Among 85 cases, 42 were classified as PS-PLA$_1$-high, and 43 as PS-PLA$_1$-low.

PS-PLA$_1$ expression and clinicopathological variables. Univariate analysis of the association between PS-PLA$_1$ expression and the clinicopathological variables are shown in Table II. The variables associated with PS-PLA$_1$ expression were tumor size (p=0.0091), tumor depth (p=0.0040) and hematogenous metastasis (p=0.0082). High levels of PS-PLA$_1$ expression were observed in cases with larger tumor size, more advanced tumor depth, and those with hematogenous metastasis. No significant correlation between PS-PLA$_1$ expression and any of the other clinicopathological variables was observed. Independent variables that correlated with expression of PS-PLA$_1$ were identified using logistic regression analysis (Table III). Greater tumor depth (p=0.039, OR=3.40) and hematogenous metastasis (p=0.040, OR=3.77) were found to independently correlate with the expression of PS-PLA$_1$.

PS-PLA$_1$ expression and prognosis of patients with CRC. The correlation between PS-PLA$_1$ expression and the prognosis of the 75 patients with CRC who had undergone curative surgery was evaluated by the Kaplan-Meier method and the log-rank test. As shown in Figure 2, the DFS was significantly shorter in patients with PS-PLA$_1$-high tumors compared to those with PS-PLA$_1$-low ones (5-year DFS rate: 74.0% *vs*. 86.9%, p=0.0087).

Correlation between the other clinicopathological variables and DFS is shown in Table IV. In the univariate analysis, more advanced tumor depth (p=0.024), presence of venous involvement (p=0.0046) and hematogenous metastasis (p<0.0001) were risk factors of poorer DFS. The presence of hematogenous metastasis, however, was the only independent risk factor associated with poor DFS when analyzed by the Cox proportional hazard model, and the expression of PS-PLA$_1$ did not serve as an independent prognostic factor (Table V).

Discussion

PS-PLA$_1$ is an enzyme responsible for the production of LysoPS, a kind of lysophospholipid mediator. Much attention has recently been paid to the lysophopholipid mediators, such as lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), which are known to be implicated in cancer development, together with the enzymes responsible for their production (autotaxin and sphingosine kinases) and their receptors (LPA and S1P receptors) (11, 12, 13, 14). In contrast to these lysophospholipid mediators, the physiological functions of LysoPS, and PS-PLA$_1$, the enzyme responsible for its production, have not yet been fully-elucidated, especially in the field of oncogenesis and tumor metastasis. To the best of our knowledge, this is the first report to demonstrate the correlation between the expression of PS-PLA$_1$ and the clinicopathological features associated with progression of CRC using immunohistochemistry.

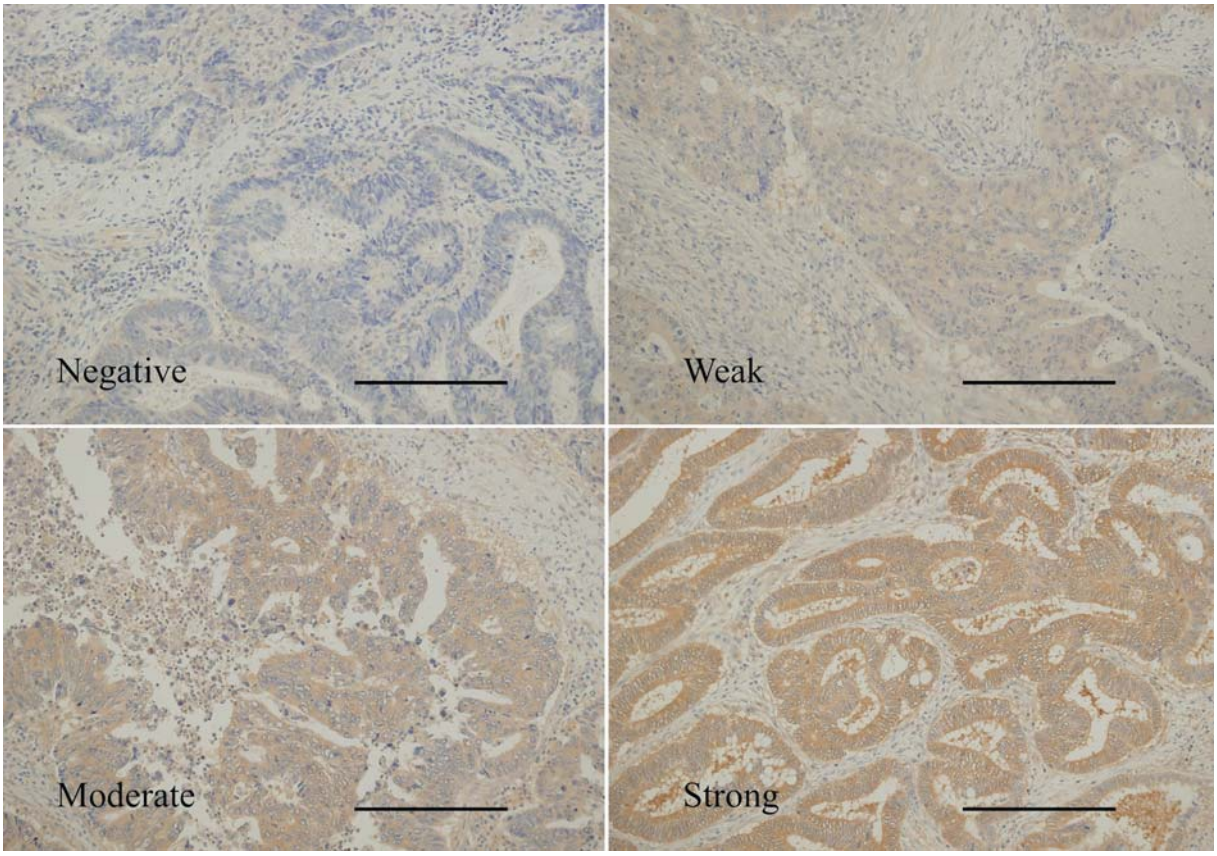

Figure 1. *Colorectal cancer sections immunostained by a monoclonal antibody against human phophtidylserine-specific phospholipase A1 (PS-PLA$_1$). PS-PLA$_1$ staining was mainly observed in the cytoplasm of cancer cells. The immunostaining intensity was classified into four grades, as follows: negative, weak, moderate, and strong. Bar=200μm.*

We found that high PS-PLA$_1$ expression was associated with tumors of deeper invasion and a higher incidence of hematogenous metastasis. In addition, we observed that PS-PLA$_1$ expression was associated with poorer DFS. These findings strongly indicate the important roles of PS-PLA$_1$ in tumor invasion and metastasis of CRC. It also raises the possibility of use of PS-PLA$_1$ as a diagnostic marker of CRC.

The most important finding of this study was that PS-PLA$_1$ expression correlated with tumor invasion and metastasis. However, the mechanism of oncogenesis or tumor metastasis promotion in CRC by PS-PLA$_1$ or LysoPS remains to be elucidated. LysoPS was reported to stimulate the chemotactic migration of a glioma cell line (5), which is compatible with our finding of the correlation of PS-PLA$_1$ expression with tumor invasion. Recently, we reported the stimulation of chemotactic migration of CRC cell lines (7), a finding that also supports our present result. PS-PLA$_1$ expression was detected in the poorly-metastatic human melanoma cell lines, but not in the highly-metastatic ones (6), suggesting that in melanoma cells, an inverse association exists between PS-PLA$_1$ expression and the metastatic potential. This finding is contradictory to our finding with CRC. Three human G protein-coupled receptors, namely GPR34, P2Y10 and GPR174, were reported to react specifically to LysoPS (15). These LysoPS receptors were found to be differentially expressed in distinct metastatic sites of melanoma. Interestingly, GPR174 was statistically significantly overexpressed in subcutaneous metastases (16). Thus, it is possible that GPR34, P2Y10 and GPR174 may induce different physiological functions in response to LysoPS. Additionally, it is possible that the expression pattern of LysoPS receptors differs between melanoma and CRC cells, leading to contradictory effects.

We showed that PS-PLA$_1$ expression was associated with poorer DFS, indicating that PS-PLA$_1$ may be an important prognostic factor in CRC. However, it was found that PS-PLA$_1$ expression significantly correlates with the presence of hematogenous metastasis, and this may have affected the association of PS-PLA$_1$ expression with poorer DFS. By the logistic regression analysis, PS-PLA$_1$ did not remain an

Table II. *Univariate analysis of the association between clinicopathological variables and phophtidylserine-specific phospholipase A1 (PS-PLA$_1$) immunstaining.*

Variable		n	PS-PLA$_1$ High (n=42)		PS-PLA$_1$ Low (n=43)		*p*-Value
Mean age (years)±SD		85	63.4±10.2		66.3±11.7		0.22
Gender	Male	51	22	(52.4%)	29	(67.4%)	0.16
	Female	34	20	(47.6%)	14	(32.6%)	
Tumor location	Colon	56	24	(57.1%)	32	(74.4%)	0.092
	Rectum	29	18	(42.9%)	11	(25.6%)	
Tumor size (mm)±SD		85	51.7±23.4		38.3±23.1		0.0091
Tumor depth[a]	T1-T2	24	6	(14.3%)	18	(41.9%)	0.0040
	T3-T4	61	36	(85.7%)	25	(58.1%)	
Histological type	Well/mod	79	41	(97.6%)	38	(88.4%)	0.20
	Muc/poor	6	1	(2.4%)	5	(11.6%)	
Lymphatic involvement	Negative	63	31	(73.8%)	32	(74.4%)	0.95
	Positive	22	11	(26.2%)	11	(25.6%)	
N factor	Negative	40	18	(42.9%)	22	(51.2%)	0.44
	Positive	45	24	(57.1%)	21	(48.8%)	
Venous involvement	Negative	35	15	(35.7%)	20	(46.5%)	0.31
	Positive	50	27	(64.3%)	23	(53.5%)	
Hematogenous metastasis	Absent	67	28	(66.7%)	39	(90.7%)	0.0082
	Present	18	14	(33.3%)	4	(9.3%)	

SD: Standard deviation; [a]TNM Classification of Malignant Tumors of the Union for International Cancer Control (UICC, 7th edition) (9).

Table III. *Multivariate logistic regression analysis of the correlation between clinicopathological variables and increased tumor immunostaining for phophtidylserine-specific phospholipase A1 (PS-PLA$_1$) in patients with colorectal cancer.*

Variable	*p*-Value	Odds ratio (95% CI)
Tumor size (≥50 mm *vs*. <50 mm)	0.79	1.16 (0.38-3.59)
Tumor depth (T3-4 *vs*. T1-2)[a]	0.039	3.40 (1.06-12.0)
Hematogenous metastasis (positive *vs*. negative)	0.040	3.77 (1.06-15.7)

CI: Confidence interval; [a]TMN Classification of Malignant Tumors of the Union for International Cancer Control (UICC, 7th edition) (9)

Table IV. *Univariate analysis of disease-free survival (DFS) of patients with colorectal cancer using log-rank test.*

Variable	5-Year DFS rate (%)	*p*-Value
PS-PLA$_1$ (high *vs*. low)	74.0 *vs*. 86.9	0.0087
Gender (male *vs*. female)	72.1 *vs*. 77.1	0.58
Tumor location (colon *vs*. rectum)	79.3 *vs*. 63.6	0.084
Tumor size (≥50 mm *vs*. <5 0mm)	62.2 *vs*. 79.7	0.086
Tumor depth (T3-4 *vs*. T1-2)[a]	65.6 *vs*. 91.7	0.024
Histological type (muc/poor *vs*. well/mod)	100 *vs*. 72.5	0.26
Lymphatic involvement (positive *vs*. negative)	57.8 *vs*. 78.8	0.073
N factor (positive *vs*. negative)	65.0 *vs*. 82.1	0.14
Venous involvement (positive *vs*. negative)	60.1 *vs*. 90.9	0.0046
Hematogenous metastasis (positive vs. negative)	20.0 *vs*. 82.5	<0.0001

PS-PLA$_1$: Phophtidylserine-specific phospholipase A$_1$; [a]TMN Classification of Malignant Tumors of the Union for International Cancer Control (UICC, 7th edition) (9).

independent prognostic factor. It is possible that PS-PLA$_1$ plays an important role as a prognostic factor when analyzed in combination with other factors. These possibilities are now being investigated in our laboratory.

PS, the sole substrate for PS-PLA$_1$, is normally restricted to the inner surface of the cellular membrane and translocates to the cellular surface in apoptotic cells and antigen-activated lymphocytes (17). PS exposure is also evident in cancer cells treated with chemotherapy or radiotherapy (18, 19), indicating that abundant LysoPS is produced by PS-PLA$_1$ under such conditions. Since chemotherapy and chemoradiotherapy are becoming standard modalities in the management of CRC, especially for those far advanced with distant metastasis, or local or distal recurrence after curative treatment (20), targeting the function of PS-PLA$_1$ could be important for the development of new therapeutic strategies.

In conclusion, we demonstrated, as far as we are aware for the first time, the association of PS-PLA$_1$ expression and the progression and prognosis of CRC. Further studies will be

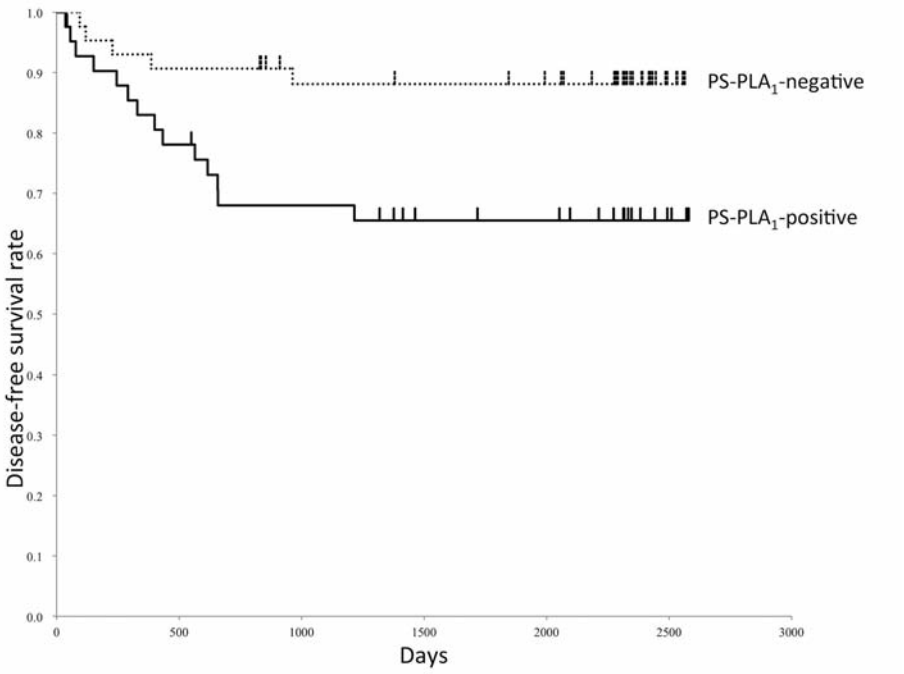

Figure 2. *The disease-free survival (DFS) rate for the 75 patients who underwent curative surgery, as evaluated by the log-rank test. The DFS rate was significantly shorter in patients with high phophtidylserine-specific phospholipase A1 (PS-PLA$_1$) immunostaining compared to those with low immunostaining.*

Table V. *Multivariate analysis of variables for disease-free survival of patients with colorectal cancer using Cox hazard model.*

	Disease-free survival	
Variable	*p*-Value	Hazard ratio (95% CI)
PS-PLA1 (high *vs*. low)	0.274	1.89 (0.611-6.64)
Tumor depth (T3-4 *vs*. T1-2)[a]	0.204	2.60 (0.625-17.8)
Venous involvement (positive *vs*. negative)	0.159	2.52 (0.708-11.8)
Hematogenous metastasis (positive *vs*. negative)	0.0033	5.09 (1.75-15.2)

CI: Confidence interval; PS-PLA$_1$: phophtidylserine-specific phospholipase A$_1$; [a]TMN Classification of Malignant Tumors of the Union for International Cancer Control (UICC, 7th edition) (9)

necessary to confirm the mechanistic events involved, not only in the progression of CRC, but also in other tumor types.

Acknowledgements

This study was supported, partly, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

The Authors thank Ms. Mika Matsuhashi, Ms. Michiru Kawabata, Mr. Yutaka Nagura and Ms. Yuuko Mishima from the Department of Transfusion Medicine and Mrs. Kaoru Amitani from the Department of Surgical Oncology (The University of Tokyo) for their kind advisory and technical assistance.

Conflicts of Interest

The Authors declare that they have no conflicts of interest with regard to this study.

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Received November 27, 2014
Revised December 9, 2014
Accepted December 12, 2014